MODULATION OF ION CHANNELS AND IONIC PUMPS BY FATTY ACIDS: IMPLICATIONS IN PHYSIOLOGY AND PATHOLOGY

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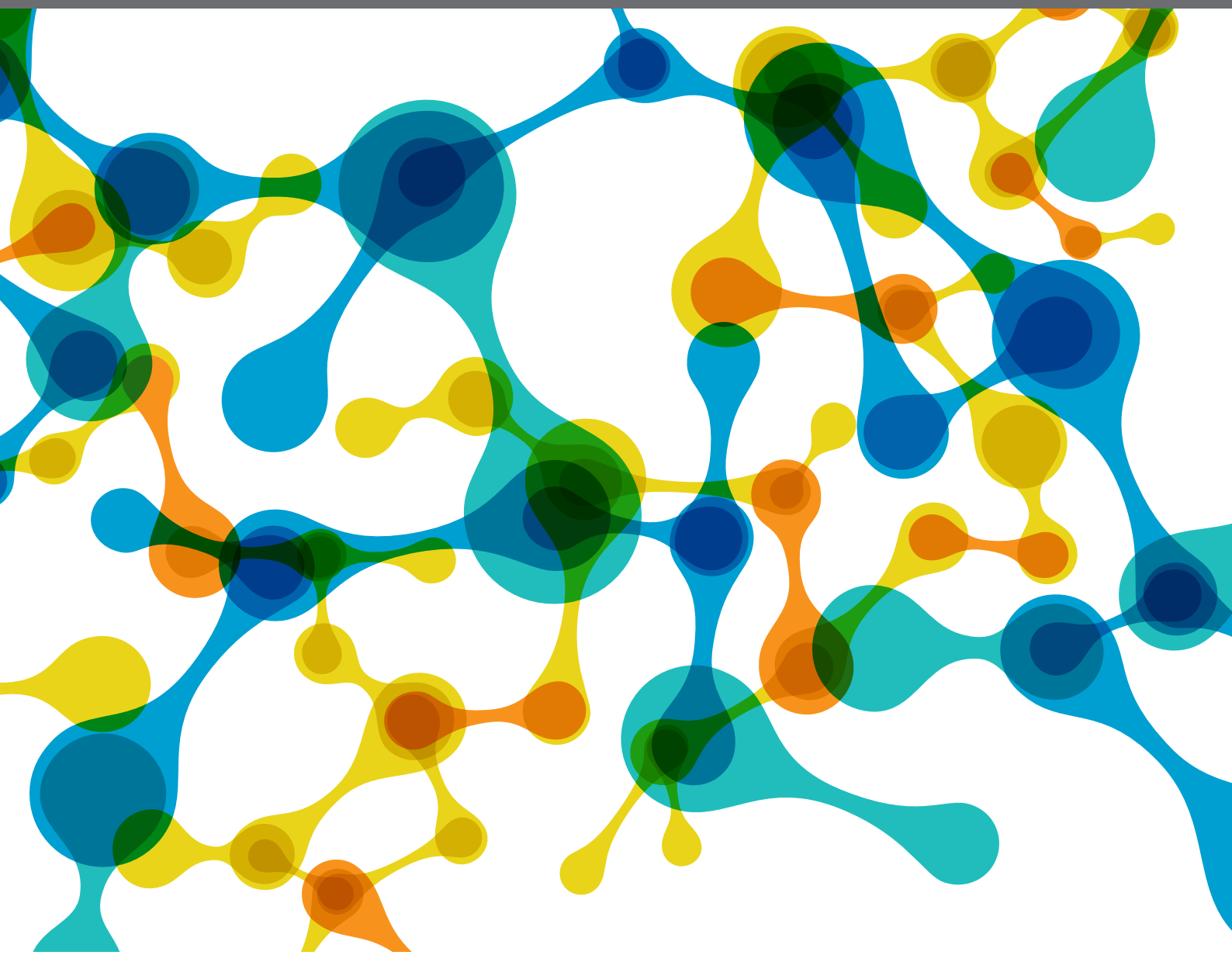

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MODULATION OF ION CHANNELS AND IONIC PUMPS BY FATTY ACIDS: IMPLICATIONS IN PHYSIOLOGY AND PATHOLOGY

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EDITORIAL
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Editorial: Modulation of Ion Channels and Ionic Pumps by Fatty Acids: Implications in Physiology and Pathology

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Editorial on the Research Topic

Modulation of Ion Channels and Ionic Pumps by Fatty Acids: Implications in Physiology and Pathology

Polyunsaturated fatty acids (PUFAs) are fatty acids with two or more unsaturations. They are essential components of membrane phospholipids and it is well-known that under physiological conditions, PUFAs have a big impact in the human health. There exist two major series of PUFAs, i.e., omega-3 (or n-3 PUFA) and omega-6 (or n-6 PUFA), depending on the positions of the first double bond. The balance between n-6 PUFA and n-3 PUFA, is critical and associated to pathological conditions such cardiovascular and neurodegenerative diseases (Díaz and Marín, 2013; Dyall, 2017; Abdelhamid et al., 2018). In general, these fatty acids are incorporated into cell membrane phospholipids and are responsible for tuning a number of physicochemical properties of the plasma membrane, but are also the source for different bioactive lipids which have different effects on human health. Further, when free radicals are produced, PUFAs can be oxidized to lipid peroxides, which have profound effects on cell function, even inducing cell death (Catalá and Díaz, 2017). In spite of all these years of research, it is still not completely understood the molecular and cellular basis that rule the effects of PUFAs and lipid-derived peroxides on different cellular processes. Mounting evidence accumulated over the last decades indicate that many of their effects may be attributed to the modulation of membrane bilayer microenvironment, e.g., microdomains dynamics and a plethora of physicochemical properties, but also through direct interactions with membrane embedded proteins, these including ion channels, ionic pumps, transporters, neurotransmitter and hormone receptors, amongst other protein entities, at the plasma membrane which, in turn, will modify cellular contents of ions and metabolites, signaling and transduction pathways impacting the many faces of cell homeostasis (Cordero-Morales and Vásquez, 2018).

In this research topic we have gathered together 10 articles which contribute to the comprehension of the effects of fatty acids (PUFAs and other bioactive lipids) on ion channels and ionic pumps properties. The focus is put not only in the molecular interactions, but also on the systemic effects of their alterations in pathological conditions, as for example in the bipolar disorder (hypothesized in the article by Riveros and Retamal), which might pave the way for their understanding and to uncover new therapeutical directions.

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In this Research topic, Antollini and Barrantes reviewed the effects of PUFAs on several types of voltage-gated K^+, Na^+, and anionic channels as well as in GABA and nicotinic receptors. Based on the current data they concluded that the effect of a given PUFA depend on the type of channel under consideration and, moreover, that similar PUFAs may cause diverse effects on very closely related channels from a phylogenetic point of view. However, it seems clear that almost all PUFAs that modulate voltage- and ligand-gated channel directly contact with them. Therefore, nowadays a hot topic in the field is the study of those amino acids that participate in the interaction with the PUFAs. Antollini and Barrantes also discuss some of the rules that govern the potential interaction between amino acid residues and PUFAs. Thus, the length, isomerism and saturation of the PUFAs seems to be very important factors. In this order of ideas, Elinder and Liin summarize data from different PUFAs on voltage-gated ion channels containing one or more voltage-sensor domains, such as voltage-gated sodium (Na_V), potassium (K_V), calcium (Ca_V), and proton (H_V) channels, as well as calcium-activated potassium (K_{Ca}), and transient receptor potential (TRP) channels. In this very interesting work they propose that the effect of PUFAs on voltage gated ion channels can be grouped into three main categories (1) those causing alterations in voltage dependence, (2) those causing changes in the maximal conductance and (3) those causing changes in the gating kinetics. They also propose five molecular mechanism by which PUFAs can exert these effects: (1) by binding to amino acids located at the ion-conducting pore, (2) by binding to amino acids at the extracellular side of the channel forming an open-channel block, (3) by binding at the voltage sensor domain (VSD) linker close to the intracellular gate, (4) at the interface between the extracellular part of the channel and the external leaflet, from where they can electrostatically affect the S4 VSD and (5) by electrostatic effects on the pore domain. In agreement, in the article by Moreno et al. on K^+ channels, the mechanism of modulation involves an electrostatic effect of PUFAs on VSD. These authors explain in detail that the negative charge of the carboxyl group of the fatty acid is crucial for the electrostatic interaction between PUFA and the VSD (at the bilayer/channel interface close to the S3-S4 segment). Similarly, in the original research article by Oliván-Viguera et al., the effects of omega-3 fatty acids on the calcium/calmodulin-gated KCa3.1 properties expressed in murine and human fibroblasts were analyzed. KCa3.1 channel function has been linked to abnormal cell proliferation, pathological tissue remodeling and fibrosis of a variety of organs, chronic inflammation, and autoimmune diseases. They found that α-linolenic acid and docosahexanenoic acid (DHA) inhibit KCa3.1 currents and strongly reduce fibroblast growth in a dose-dependent way. The association between KCa3.1 and PUFA as well as the link between KCa3.1 and human diseases, led them to suggest that reduced fibroblast KCa3.1 functions might be a feature and a possible biomarker of cell dysfunction in Lysosomal Storage Disorders.

PUFAs can also modulate ion channels activity through generation of bioactive lipids. In particular pro-resolvins have been shown to modulate different types of ion channels, including epithelial Na^+ channels, cystic fibrosis transmembrane conductance regulators, ATP-sensitive K^+ channels, Ca^{2+}-activated anion channels, pannexin 1 hemichannels, and canonical subtype of TRP channels (TRPCs). In this RT, Choi and Hwang review the effects of various pro-resolving lipids on the functions of four sensory TRP channels and NMDA receptors and discussed their beneficial effects on inflammation and pain. Nowadays, the physiological significance of bioactive lipids is expanding and new roles in their signaling properties are growing rather slowly, perhaps, the main reason for this, is that these molecules are usually found in very small quantities and often they are co-extracted with other lipophilic molecules, making their detection and identification a hard task. In this RT, Divito et al. discuss on the fact that common analytical methodologies are usually ineffective due to the unsuccessful separation of bioactive lipids in complex biological samples, and show that novel multidimensional separation techniques are very useful separation strategies for these elusive molecules.

Several articles are included in this RT showing different forms of regulation of Cx46 hemichannels by lipid molecules. In this Research Topic, Retamal presents evidence supporting the hypothesis that carbon monoxide inhibits Cx46 hemichannels through a lipid peroxide-dependent process. Hemichannels are ion channels composed of six protein subunits known as connexins (Cxs). Under physiological conditions they present a low open probability however, massive and/or prolonged hemichannel opening induces or accelerates cell death. It is known that carbon monoxide (CO) modulates many cellular processes through the activation of guanylate cyclase and/or inducing direct carbonylation of proteins by mechanisms that are either sensitive or insensitive to reducing agents. Additionally, in the article by Puebla et al. the effects of PUFA on Cx hemichannels, gap junction channels (GJCs), and channels formed by Pannexins (Panx) are reviewed. Panx as Cxs channels are permeable to large molecules such as ATP and glutamate. The authors show that PUFAs can act as either inhibitors or activators of Cxs and Panxs, and these opposing effects can be mediated by different post-translational modifications that affect these two types of proteins.

Using a different strategy, Diaz et al. have analyzed the effect of diets containing different PUFAs composition on the thermodynamic properties of Na^+-K^+-ATPase and on the lipid composition of gill epithelia and isolated enterocytes from euryhaline teleost fish. They demonstrate not only that branchial cells and enterocytes have different lipid composition, but also that they differently respond to severe PUFAs-deficient diets, unraveling powerful lipostatic mechanisms protecting membrane lipids in branchial cells. Paralleling these results, the thermodynamic properties of the Na^+-K^+-ATPase were modified in enterocytes but not in branchial cells. They discuss on direct and specific interactions of different fatty acid-containing phospholipids and cholesterol within the Na^+-K^+-ATPase molecular framework, determining the thermodynamic traits and enzyme activity within a given lipid microenvironment.

The original idea of this Research topic was to broaden the interest of the scientific community in studying the relationship between fatty acids (and derivatives) and integral membrane proteins. We expect this compilation may stimulate scientists

to endeavor research aimed at uncovering the molecular mechanisms underlying their interaction and how it is involved in regulation/dysregulation of cell function under normal and pathological conditions.

AUTHOR CONTRIBUTIONS

MD and MAR have edited the research topic and have written and drafted this editorial.

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REVIEW
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Are Polyunsaturated Fatty Acids Implicated in Histaminergic Dysregulation in Bipolar Disorder?: AN HYPOTHESIS

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Bipolar disorder (BD) is an extremely disabling psychiatric disease, characterized by alternate states of mania (or hypomania) and depression with euthymic states in between. Currently, patients receive pharmacological treatment with mood stabilizers, antipsychotics, and antidepressants. Unfortunately, not all patients respond well to this type of treatment. Bipolar patients are also more prone to heart and metabolic diseases as well as a higher risk of suicide compared to the healthy population. For a correct brain function is indispensable a right protein and lipids (e.g., fatty acids) balance. In particular, the amount of fatty acids in the brain corresponds to a 50–70% of the dry weight. It has been reported that in specific brain regions of BD patients there is a reduction in the content of unsaturated n-3 fatty acids. Accordingly, a diet rich in n-3 fatty acids has beneficial effects in BD patients, while their absence or high levels of saturated fatty acids in the diet are correlated to the risk of developing the disease. On the other hand, the histamine system is likely to be involved in the pathophysiology of several psychiatric diseases such as BD. Histamine is a neuromodulator involved in arousal, motivation, and energy balance; drugs acting on the histamine receptor H3 have shown potential as antidepressants and antipsychotics. The histaminergic system as other neurotransmission systems can be altered by fatty acid membrane composition. The purpose of this review is to explore how polyunsaturated fatty acids content alterations are related to the histaminergic system modulation and their impact in BD pathophysiology.

Keywords: BAS, bipolar disorder, fatty acids, histaminergic system, omega-3, PUFAs

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INTRODUCTION

Bipolar disorder (BD) is a prevalent psychiatric disease characterized by recurrent episodes of depression and elevated mood (mania), intermingled with periods of normal mood (euthymia) (Phillips and Kupfer, 2013). Pharmacological treatments consist of lithium for prophylaxis, as well as anticonvulsants and antipsychotics for acute treatment in manic episodes, in conjunction with

psychotherapy (Geddes and Miklowitz, 2013). All these pharmacological approaches have side effects that negatively impact on the life quality of patients. Is well-recognized that BD constitutes a significant burden for the patient (reduced life expectancy and increased risk of suicide), because of that considerable amount of efforts has been conducted in this field in order to improve the patient's quality of life (Vogelzang et al., 2012). One major problem to find new and better treatments for BD symptoms is the lack of a good animal model that recapitulates the core feature of this disorder: spontaneous oscillations between manic, euthymic, and depressive-like behavioral phenotypes. Currently, most of the research is based on animal models that show manic-like behavior without depression. Nevertheless, BD patients spend more time in the depressed phase than in their manic phases (Judd et al., 2002). Depression in BD is usually refractory to treatments and has to be treated differently than monopolar depression, which uses drugs like tricyclic antidepressants because these can trigger the switch from depression to mania (Gijsman et al., 2004). It would be crucial to differentiate what distinguishes bipolar depressed from a monopolar depressed state and which are the alterations that lead to switching from depressed to manic state and destabilize the euthymic state in BD patients. BD has a strong genetic component being transmitted within families for several generations (Craddock and Sklar, 2013). Genetic alterations for BD have contributed to understanding the characteristic features of the disease, and have helped to determine that BD shares a wide range of features with psychiatric diseases such as autism, monopolar depression, and schizophrenia. The genetic bases of this disease seem to be multifactorial instead of caused by a single gene mutation. However, in spite of this genetic component, this disease can be drastically modulated by the fat diet composition, been unsaturated fatty acids (e.g., omega-3) diet composition crucial. Additionally, dysfunctions in the neuromodulator systems are also common in various psychiatric disorders. Thus, extensive research about the relationship between serotonin, noradrenaline, and dopamine on different mental diseases has been done, but the role of histamine in psychiatric illnesses has been much less studied (Baronio et al., 2014). Moreover, the link between the histaminergic system and fatty acids appears to have a lot of therapeutic potential against BD. In this review, we will discuss the recent evidence regarding the link between fatty acid intake, histaminergic system modulation, and the BD improvements.

FATTY ACIDS AND BIPOLAR DISORDER

The amount of fatty acids in the brain corresponds to a 50–70% of its dry weight (O'Brien and Sampson, 1965b), and the composition of fatty acids is very important for its right function. In particular, polyunsaturated fatty acids (PUFAs) correspond to 20% of brain weight (O'Brien and Sampson, 1965a), and among them, docosahexaenoic acid (DHA, C22:6n-3) and arachidonic acid (AA, C20:4n-6) are the most abundant (O'Brien and Sampson, 1965a). Both, AA and DHA (and their metabolites), participate in many important brain functions such as acting as intracellular second messengers, neurotransmission, gene transcription, among other brain processes (Hibbeln et al., 1989). Additionally, fatty acids at the cell membrane can directly interact with membrane proteins, determining their structure and function. Thus, they can determine the membranes fluidity, lateral pressure, bilayer thickness, and surface charge distribution (Stillwell and Wassall, 2003). There is a high specificity in lipid–protein interaction, for example, slight changes in fatty acid conformation can alter their interaction with proteins, as for example the loss of one double bond when a molecular simulation is conducted replacing DHAn-3 with DPAn-3, generate changes in the balance of attraction and repulsion forces that determinate the bending force in a monolayer which is translated in the lateral pressure profile in a bilayer which can alter protein functional conformational changes as for example G protein-coupled receptors activation (Gawrisch and Soubias, 2008). Therefore, the presence of DHA in the membrane can modulate neurotransmission systems signaling, contributing in this way to brain function. Moreover, PUFAs can affect brain functioning by activating many kinds of receptors and cell signaling pathways and also by modulating the endocannabinoid system. These potential mechanisms of action of n-3 fatty acids in brain physiology have been revised in detail by Bazinet and Layé (2014).

Accordingly, to the exposed above, epidemiological studies have revealed that fatty acids composition of the diet is associated with mental health (Messamore et al., 2017). As for example, populations with a lower incidence of BD have higher rates of seafood consumption (Noguchi et al., 2013). While a study comparing intake of PUFAs in bipolar patients versus a non-psychiatric control population showed that bipolar diagnosed individuals have a reduced intake of PUFAs [eicosapentaenoic acid (EPA) (n-3), docosahexaenoic acid (DHA) (n-3), arachidonic acid (AA) (n-6), and docosapentaenoic acid (DPA)] and higher intake of saturated fats (Evans et al., 2014). Moreover, not just the saturated versus PUFAs consumption is relevant, but also very important is the ratio among n-3 and n-6 PUFAs consumption, for more details about this topic (see Messamore et al., 2017). Nevertheless, diet composition does not constitute an estimate of the fatty acid composition in the brain, in order to establish a functional relation of fatty acid composition and brain physiology alterations an estimation of brain PUFAs content is needed. A non-invasive way to estimate brain fatty acid composition is the erythrocyte fatty acid composition, because erythrocyte DHA and cortical DHA content are correlated (Carver et al., 2001). The amount of DHA measured in the plasma membranes of erythrocytes in BD patients is lower compared to the DHA in control patients (Chiu et al., 2003; McNamara and Welge, 2016). These results are in agreement with animal studies showing that diet deficient in n-3 fatty acids alter monoamine systems in limbic structures known to control mood (Chalon, 2006; Jiang et al., 2012), which provides a possible link between fatty acid intake and psychiatric disorders as BD.

Moreover, fatty acids in the brain could be linked to neuroinflammatory processes, known to be at the base of many

psychiatric diseases and BD. AA is an n-6 PUFA which initiates a signaling cascade by conversion of a part of the released AA to bioactive eicosanoids. Cyclooxygenase 2 (COX2) transforms AA into the prostaglandin E2 (PGE2) initiating neuroinflammation processes (Rapoport, 2008). Post-mortem brains of BD patients have increased levels of AA synthesizing enzymes (Kim et al., 2011). It has been proposed an AA hypothesis to explain the effect of mood stabilizers used to treat BD. Mood stabilizers affect AA cascade but not in a single point of action, for example, lithium and carbamazepine decrease AA levels by reducing PLA2 expression, and therefore AA levels. Additionally, downstream signaling of AA is reduced by lithium, carbamazepine, valproate, and lamotrigine because they inhibit COX2 expression. AA signaling cascade as a target for BD symptomatology treatment could also help to explain the positive action of an n-3 fatty acid enriched diet in BD, because n-3 fatty acids oppositely regulate AA and DHA bioavailability (Rao et al., 2007) they avoid overreactions in AA cascade (Lands, 2015). Accordingly, antidepressants with high risk to produce mania as fluoxetine and imipramine increase brain AA concentration and cPLA2 activity (Rao et al., 2006; Lee et al., 2010), but bupropion or lamotrigine which do not induce AA production, are not associated to the risk of inducing mania.

It has been recently reported using molecular simulation that the presence of DHA in the membrane accelerates the rate of oligomerization of dopamine D2 receptor and adenosine A2A receptor, being higher when the DHA content in the membrane is "healthy like" compared to a "disease like" reduced content of DHA in the membrane (Guixà-González et al., 2016). This effect is mediated by increased lateral mobility of receptors and favorable interaction between DHA and proteins as well as favorable interactions among DHA tails and the rest of the membrane lipids, which induces segregation of DHA-coated proteins in enriched DHA domains. Therefore, the increased diffusion and concentration of proteins in these domains increases the chances of protein–protein interactions and accelerates oligomerization (Guixà-González et al., 2016). Nevertheless, information regarding the effect other PUFA as for example n-6 PUFA on protein–protein interaction is lacking, in order to link this molecular effect of DHA on dopamine and adenosine oligomerization to behavioral effects related to n-3/n-6 ratio it would be necessary to evaluate the effect of membrane enrichment with an n-6 PUFA.

In animal models, the use of controlled oil supplementation in the diet has been extremely helpful to elucidate how lipid composition impacts BD related symptomatology. For example, supplementation of the diet for two generations with either fish oil (rich in n-3 fatty acids) or a diet rich in trans fatty acids (TFA) from pregnancy to adulthood, induced different brain lipid composition in the second generation (Trevizol et al., 2015). The second generation of rats fed a diet rich in TFA had a reduced n-3 fatty acids composition in the brain, together with an increased hyperactivity response to amphetamine injection compared to fish oil group. The brains of fish oil supplemented animals have also higher levels of BDNF and reduced reactive oxygen species (ROS) compared to animals fed with the TFA rich diet, showing that a high level of trans-fatty acids in the diet may facilitate the development of neuropsychiatric conditions, including BD. Moreover, a diet high in TFA resulted in a reduced expression of dopamine transporter in the hippocampus and increased rates of amphetamine self-administration, in the second generation of rats (Trevizol et al., 2015). Fatty acids composition of the brain can also be related to addictive behavior which is notably higher in BD population (Regier et al., 1990). These data suggest that symptomatology of BD, as well as altered pathways involved in it, could be modulated by diet supplementation with PUFAs, and the data emphasize the important role of PUFAs in BD etiology.

Similarly, to the example exposed above, chronic amphetamine administration in mice, induces an increased locomotor response to amphetamine after withdrawal, this is known as sensitization. Sensitized mice have manic and depressive behaviors together with circuitry alterations related to BD (Pathak et al., 2015). In accordance, an increase in the AA:DHA ratio induced by the dietary deficiency in DHA, increases the behavioral sensitization to amphetamine (McNamara et al., 2008). AA:DHA ratio positively correlates with amphetamine-induced locomotor activity, DHA deficiency also induces an increase in DHA extracellular concentration in the brain that is positively correlated with AA:DHA ratio and amphetamine-induced locomotor activity (McNamara et al., 2008).

Furthermore, trials using n-3 PUFA diet enrichment have shown variable but promising results for the treatment of BD. For example, supplementation with n-3 PUFA as adjunctive therapy has a positive effect in bipolar depression, significantly bigger than placebo, but the same methanalysis concludes that this is not true for manic symptoms (Sarris et al., 2012). However, some reports show less confident results, as for example the metanalysis performed by Montgomery et al showing that the design and execution of the trials must be improved in order to reach conclusive results, for example, the duration of the trial has to be of minimum 3 months to achieve changes in brain fatty acids composition, the conclusion of this metanalysis is that of the analyzed works just one have positive results but just in depressive symptoms, again, with no effect of n-3 supplementation on manic symptoms (Montgomery and Richardson, 2008).

Moreover, caution should be taken when interpreting results of n-3 diet enrichment in patients and designing trials. One of the issues to take in account is the impact of the disease in the patient's style of life. BD diagnosed population has higher rates of unemployment and divorce, familial conflicts, and poor diet habits (Morselli et al., 2004). Considering this, just to adhere to a trial could have a positive effect, because of the increase in the structure in their lifestyle. It should also be considered, that omega-3 supplementation can have a positive effect in overall health, as it has been reported to be beneficial for metabolic alterations and cardiovascular health (Carpentier et al., 2006; Sudheendran et al., 2010). Therefore, it should be taken in to account that together with their effect on brain functioning omega-3 supplementation could improve bipolar symptoms trough by increasing well-being and improving cellular and systemic functioning in general.

A better understanding of the consequences of n-3/n-6 ratio in BD symptomatology could help to improve treatments targeting lipid composition and its molecular consequences.

HISTAMINERGIC NEUROTRANSMISSION SYSTEM

Neuronal histamine is synthesized by neurons in the tuberomammillary nucleus (TMN) of the posterior hypothalamus (Brown et al., 2001). In mammalian brain, histamine acts (mainly) as an excitatory neurotransmitter and has been implicated in various central functions, as for example, arousal modulation, motivation, energy balance, motor behavior, and cognition (Benarroch, 2010; Torrealba et al., 2012). The histaminergic system has been implicated in energy balance in many ways (Tabarean, 2016). For example, the regulation of food consumption, energy expenditure (Yoshimatsu, 2006) and thermoregulation (Tabarean et al., 2012). Histamine effects are mediated by the activation of four G protein-coupled receptors: H1R, H2R, H3R, and H4R (Panula and Nuutinen, 2013). H1R and H2R activation have excitatory effects in post-synaptic neurons (Haas et al., 2008). H3R is the most abundant in the CNS, and it can be either an autoreceptor, located on the dendrites and axonal varicosities of histaminergic neurons, where it modulates the release of histamine or it can be a heteroreceptor able to regulate the release of other neurotransmitters. In addition, H4R is expressed mainly on immune cells, and it modulates the immune response during the inflammatory state (Deesch et al., 2005). In the CNS the function and location of H4Rs are not completely clear (Schneider and Seifert, 2016). Histamine acting through H1 and H2 receptors increases arousal, most likely by increasing cortical and thalamic activity, as well as increasing the activity of other arousal nuclei as the orexinergic lateral hypothalamic nucleus, the noradrenergic locus coeruleus, or the cholinergic nuclei in the basal forebrain and the pons (Khateb et al., 1990). Histaminergic neurons activity is strongly correlated to active waking and their activity is drastically reduced during slow wave sleep (Takahashi et al., 2006). During awakening histaminergic signaling increase levels of arousal necessary to increase performance during goal-directed behavior, increasing vigor and persistence, as part of a motivated state. During the active phase, these histaminergic neurons promote a higher activity and excitability in cortex and thalamus, regulating, in turn, motor activity, facilitating, accelerating, and improving motor responses. For example, motor balance and coordination are modulated by histaminergic inputs to the cerebellum, acting via H2 receptors, directly in the cerebellar nuclei, the output of the cerebellum (Zhang et al., 2016). Also, histamine seems to modulate central vestibular-mediated motor reflexes and behaviors through H2 receptors in the lateral vestibular nucleus neurons which control muscle tone and vestibular reflexes, injection of histamine in this site improves motor behavior (Li et al., 2016). Furthermore, the basal ganglia, a functional network implicated in motor control, receives histaminergic projections, and histamine release during the wake period (Cumming et al., 1991), a component of the basal ganglia particularly well-innervated by histaminergic fibers is the striatum, which expresses a high density of histamine receptors (Hill and Young, 1980) supporting the idea of an important histaminergic modulation of striatal, and therefore motor, function. Motor abnormalities are part of the neurological soft signs present in BD euthymic patients, and motor hyperactivity is characteristic of mania as motor retardation characterizes depression. Thus, motor function is affected in all phases of the disease and histaminergic alterations could underlie these abnormalities.

It is interesting to note that mast cells, that promote inflammation and allergic response by releasing histamine, are also present in the brain and mast cells degranulation and non-neuronal histamine release normally promote wakefulness and motivated behavior as suggested by the increased delta power and reduced food seeking motivated behavior in mast cell-deficient mice compared to wild-types, importantly this mice have also more anxious and depressive behaviors than wild-type mice (Chikahisa et al., 2013). It is known that n-3 PUFAs have an antiallergenic effect (Willemsen, 2016) and as mentioned before, can improve BD symptoms, it is possible that both effects are related to the fact that mast cell activation is inhibited by n-3 PUFAs (Wang et al., 2015). Similarly, vitamin D that has also been suggested to improve BD symptoms (Sikoglu et al., 2015) participates in mast cell stabilization (Liu et al., 2017).

Histamine H3R are a key factor in histaminergic functions because they exert an autoinhibitory effect over histaminergic cells, controlling their activity and histaminergic release and synthesis. Thus, histaminergic neurons firing can be inhibited by H3 agonists injected into the TMN (Flik et al., 2011) The H3R mediated histaminergic autoinhibitory effect depends on calcium release from intracellular stores; in fact, preventing intracellular calcium storage uploading with thapsigargin reduce H3R mediated autoinhibition of firing frequency (De Luca et al., 2016). Also, the autoinhibitory effect of histamine through H3R is impaired in depolarized cells (De Luca et al., 2016). In addition, depolarization of histaminergic neurons increases intracellular calcium through voltage-gated calcium channels (Uteshev and Knot, 2005). H3R has been suggested as a target for drugs to treat some aspects of neurodegenerative and psychiatric diseases as cognitive dysfunctions, sleepiness, or overweight (Sander et al., 2008). Interestingly, H3 antagonists are promising as potential antipsychotic drugs (Mahmood, 2016). Accordingly, clobenpropit an H3 antagonist injected systemically or into the hippocampus reverse the immobility and cognitive impairment in a rat depression model, and this effect depends on histamine release and action over H1 and H2 (Femenía et al., 2015). Thus, the histaminergic system is promising as a target for treating depressive and manic states in BD.

Additionally, histamine through H3 can inhibit melanin-concentrating hormone (MCH) (Parks et al., 2014). MCH has been implicated in depression because injections of MCH in the dorsal raphe induce a depressive phenotype in rats (Lagos et al., 2011), that could be due to the inhibition of serotoninergic neurons by MCH (Torterolo et al., 2015). Accordingly, MCH receptor 1 antagonists have antidepressant actions (Borowsky et al., 2002). On the other hand, MCH is also related to sleep, and its levels are higher during sleep and reduced in active

wakefulness, as shown in rodents (Pelluru et al., 2013) and in humans (Blouin et al., 2013). Moreover, MCH microinjections in different areas can induce sleep and reduce latency to REM, and increase the length of REM periods, in rats and cats (Torterolo et al., 2009; Monti et al., 2015). These characteristics are similar to characteristics of sleep in depression (Palagini et al., 2013). Thus, through its action in the MCH system by H3 activation, high levels of histamine can reduce sleep, and low levels of histamine could be related to an increased MCH activity and depressive behavior. Therefore, reduction in histamine levels could induce a depressed state, by increasing MCH together with the putative impairment in histamine-derived functions such as motor activity, motivated arousal, cognitive functions, and others.

Another aspect of histamine that merits attention regarding a possible role for a histaminergic alteration in BD is the role of histamine in addiction and in particular its role in alcoholism. There is a very important comorbidity between BD and alcoholism: 50% of BD patients are alcoholics (Frye and Salloum, 2006). Rats bred for alcohol preference, have a denser histaminergic innervation through the brain, and higher histamine levels than non-alcohol-preferring rats (Lintunen et al., 2001). Also, alcohol-preferring rats have a reduced expression of H3 receptors in their motor cortex, nucleus accumbens, and CA1 area of the hippocampus (Lintunen et al., 2001). In a comparable way, post-mortem brains of BD patients have a reduced H3 expression in the hippocampus (Jin et al., 2009). Response of H3 expression to stress, sleep deprivation, inflammation, and oxidative stress is worthy of exploration, as well as the response of alcoholic animals to amphetamine in order to evaluate whether the genetic predisposition to alcohol ingestion and related alterations in the histaminergic system could be associated with a genetic predisposition to develop BD or similar disorder.

As mentioned, the histaminergic neurotransmission system is implicated in arousal and behavioral activation and is necessary for a normal unfolding of motivated behaviors. Bipolar patients have an altered response to motivational stimuli. It has been hypothesized that the extreme fluctuations in behavioral activation that patients have between mania and depression is related to a dysregulated and hypersensitive behavioral approach system (BAS) (Urošević et al., 2008). BAS is a system related to behavioral activation for the approach to reward; the prefrontal cortex has an essential role in this system, and it is functionally altered in BD. One of the PFC reported to be altered in bipolar patients is the subgenual region, which has an increased metabolic activity during mania and a decreased metabolic activity during depression (Drevets et al., 1997). Interestingly, this region corresponds to the infralimbic area of the prefrontal cortex (ILC), which is the main excitatory input to the TMN. ILC activation induces histamine release, and the increase of arousal during motivated behaviors, such as food searching behavior, depends on the increase in histamine release directed by the ILC (Riveros et al., 2015). Thus, the behavioral alterations observed in BD as excessive engagement in motivated behaviors and arousal during mania, as well as the decreased motivation and arousal during depression, could be explained by alterations in histamine transmission as well as by the functional changes in regions involved in the disease.

PROPOSED MODEL FOR FATTY ACID-INDUCED PROTECTION OF ALTERATIONS IN HISTAMINERGIC TRANSMISSION SYSTEM UNDERLYING BIPOLAR DISORDER

As mentioned before, the histaminergic system has been implicated in energy balance, food consumption, energy expenditure, and thermoregulation. Mitochondria and Na^+/K^+ ATPase activity are the main controllers of energy availability in the cells. The interplay between the histaminergic system and these players is therefore very likely to influence the energy expenditure in BD patients.

Alterations in the histaminergic system can lead to BD development and/or its worsening and an increase in PUFAs intake could reduce the vulnerability to develop BD. Here, we proposed that the protective effect of PUFAs could be mediated by the modulation of mitochondrial function and Na^+/K^+ ATPase activity in the histaminergic system (**Figure 1**).

The Na^+/K^+ ATPase Role in Mood Disorders

Na^+/K^+ ATPase is a protein that actively transports three molecules of Na^+ and two molecules of K^+ against its concentration gradient and, therefore, is particularly determinant in setting the level of neural activity. Its activity can be increased by inflammation in peripheral neurons (Wang et al., 2015), and it has been suggested that NA^+/K^+ ATPase dysfunctions may be involved in mood disorders (Christo and el-Mallakh, 1993; Traub and Lichtstein, 2000). For instance, the Na^+/K^+ ATPase activity in schizophrenic patients is diminished in the erythrocyte membrane of both unipolar and bipolar depressed patients, compared to healthy controls (Rybakowski and Lehmann, 1994). These results showed no differences between bipolar and unipolar depressed patients suggesting that alterations in Na^+/K^+ ATPase are not an endophenotype for BD, but more likely a general marker of mental health risk (Rybakowski and Lehmann, 1994). However, BD patients erythrocytes have a lower level of activity of NA^+/K^+ ATPase in manic and depressed states compared to euthymic BD patients (Looney and el-Mallakh, 1997).

Furthermore, post-mortem brains of bipolar individuals have reduced expression of Na^+/K^+ ATPase α2 in the temporal cortex (Rose et al., 1998). Accordingly, seem to be genetic associations between BD and variants encoding Na^+/K^+ ATPase α1, α2, and α3 subunits (Goldstein et al., 2009). Additionally, Myshkin (Atp1a3Myk/+; Myk/+) mice that carry a mutation in the Atp1a3 gene, results in a 36–42% reduction in total Na^+/K^+ ATPase activity in the brain (Clapcote et al., 2009), shown behavioral alterations comparable to mania [hyperactivity, risk-taking behaviors, and reductions in rapid eye movement (REM) and non-REM sleep] (Kirshenbaum et al., 2011). Furthermore,

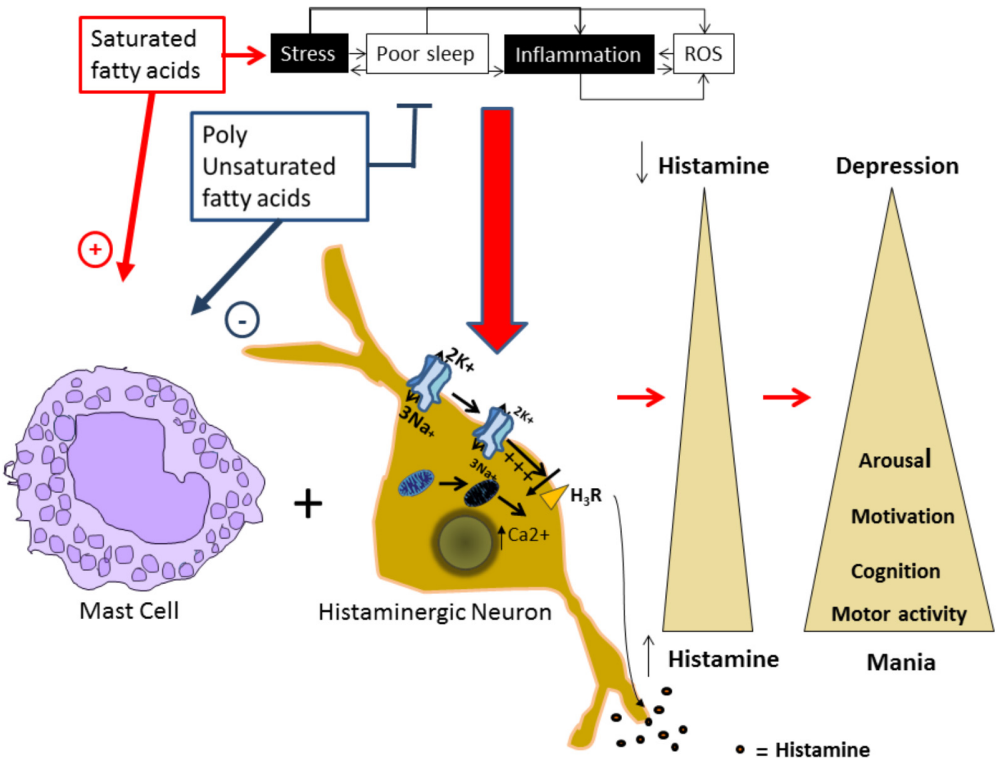

FIGURE 1 | Proposed model for alterations in the histaminergic transmission system underlying bipolar disorder. Factors as stress, inflammation, increased levels of reactive oxygen species (ROS), and sleep alterations are known to induce and or worsen BD episodes, and are risk factors for developing the disorder. In our proposed model, these factors can induce a molecular environment that can increase intracellular Ca^{2+} levels and depolarize the plasmatic membrane in histaminergic neurons. In turn, these processes could affect the H3 histaminergic auto receptor affecting the autoregulation of Histaminergic neurons, reducing Na^+/K^+ ATPase activity and inducing mitochondrial damage. This damage leads to intracellular Ca^+ increase and membrane depolarization that inhibits the H3 receptor, leading to an increased HA release. Additionally, saturated fatty acids promote mast cells degranulation and consequently histamine release, while polyunsaturated fatty acids (PUFAs) inhibit it. Released histamine by both means, acting on it targets will induce increased motor activity, arousal, and motivated behaviors, characteristic from a manic phenotype. On the contrary, all these behaviors will be reduced in depression in parallel with reduced histamine levels.

these alterations respond to lithium and valproic acid treatment (Kirshenbaum et al., 2011), suggesting that reduced Na^+/K^+ ATPase activity could be a common mechanism in behavioral abnormalities and bipolar subjects that respond to lithium and valporic acid.

The over activated state observed in BD can also be induced pharmacologically by blocking the Na^+/K^+ ATPase. Ouabain is a digitalis-like compound (DLC) that bounds to Na^+/K^+ ATPase inhibiting the transport of Na^+ and K^+ across the plasma membrane (Nesher et al., 2007). Altering Na^+/K^+ ATPase activity in the brain with an intracerebroventricular (ICV) injection of ouabain induces hyperactivity in rats (Brüning et al., 2012). This hyperactivity is reduced by administration of mood-stabilizing drugs or antipsychotics (El-Mallakh et al., 2006). Additionally, ouabain induces the release of synaptosomes from the cerebral cortex (Bersier et al., 2005). The (*n*-methyl-D-aspartate) NMDA receptor inhibitor MK-801 inhibits the ouabain-induced neurotransmitter release, suggesting that ouabain action could be mediated by the activation of NMDA receptors (Cousin et al., 1995). Furthermore, the expression of NMDA receptor subunits NR2A, NR2B, and NR2D in the cerebral cortex and hippocampus is increased by inhibition of Na^+/K^+ ATPase (Bersier et al., 2008), and an up-regulation of NMDA-evoked current in rat hippocampus neurons has been shown (Zhang et al., 2012). Ouabain inhibits glutamate uptake, because its transport depends on the Na^+ and K^+ gradients generated by Na^+/K^+ ATPase, and glutamate transporter physically interacts with Na^+/K^+ ATPase to regulate glutamatergic transmission (Rose et al., 2009) and in consequence, ouabain enhances glutamatergic neurotransmission (Nguyen et al., 2010). NMDA receptor activity regulates signal pathways related to protein translation, including ERK1/2, Akt, and mTOR, which play critical roles in synaptic plasticity regulated by the glutamatergic system (Sutton and Chandler, 2002; Gong et al., 2006). Ouabain-induced activation of mTOR signal pathway and protein synthesis could be mediated by the activation of NMDA receptor system. Ouabain injected in the brain also induces oxidative stress (Riegel et al., 2010), which is related to mood disorders, inhibits citrate synthase activity (Freitas et al., 2010), and reduces the expression of brain derived neurotropic factor (BDNF) (Jornada et al., 2010). Furthermore, ouabain activates tyrosine hydroxylase (Yu et al., 2011). All these responses resemble some aspects of the pathophysiology of mania. Therefore, injection of ouabain in brains of rodents has

been established as an accepted protocol to induce behavioral and physiological alterations used as a model of mania (el-Mallakh et al., 1995).

Effects of PUFAs on Na^+/K^+ ATPase Activity and Its Relationship With Mood Disorders

The unsaturated/saturated fatty acid ratio in the brain, has been observed to modulate Na^+/K^+ ATPase activity, being higher in animals fed with oils rich in unsaturated fats compared with animals fed with a diet reduced in unsaturated fats (Srinivasarao et al., 1997). Thus, for example, the Na^+/K^+ ATPase activity is altered by diabetes and can be partially restored after a fish oil diet (rich in n-3 fatty acids) (Gerbi et al., 1998). Accordingly, to these results, it has been shown that TFAs in the diet increases membrane rigidity and reduces the activity of Na^+/K^+ ATPase in the striatum of rats (Dias et al., 2015; Trevizol et al., 2015). Additionally, it has been observed that affinity of the α1 isoform for ouabain positively correlates with the total amount of n-6 fatty acids (Gerbi et al., 1999). These results suggest an important interaction between membrane fatty acids composition and Na^+/K^+ ATPase activity. Furthermore, the n3/n6 ratio has consequences in cognitive functions, as for example, rats fed with safflower oil which is high linoleate (n-6) have cognitive impairments when compared with rats fed the perilla oil which is high in alphalinoleate (n-3) (Yamamoto et al., 1987). This may be related to altered lipid composition of membranes and reduced Na^+/K^+ ATPase activity, in safflower oil fed rats (Tsutsumi et al., 1995). In the same line of evidence, rats fed with linseed oil show higher levels of DHA in the synaptic membranes of the brain and therefore an increased n-3/n-6 fatty acid ratio. This ratio with a higher proportion of n-3 fatty acids increases membrane fluidity, Na^+/K^+ ATPase activity, and serotonin levels in the brain (Sugasini and Lokesh, 2015). Fluidity is not the only physical property of the membrane affected by lipid composition that can have an effect on Na^+/K^+ ATPase activity. For example, the hydrophobic thickness of the membrane is also an important parameter in the pump function (Cornelius, 2001). It is determined by the carbon number in the acyl chain but also depends on the saturation of the fatty acids and their interaction with cholesterol (Cornelius, 2001), so the saturated/unsaturated ratio can also disturb the matching of the hydrophobic portion of the protein with the hydrophobic thickness of the membrane affecting by this way the pump activity (Cornelius, 2008).

It is important to note that the inclusion of PUFAs in the diet can affect neurotransmission by modulating neurotransmitter reuptake and improves the cholinergic transmission in the brain, that has an important role in cognition (Willis et al., 2009). The fatty acids composition of the diet can influence behavior and furthermore have an impact in BD symptomatology and development (**Figure 2**). Contrary to the effects of consuming PUFAs, a diet high in saturated fats is a risk factor for various mental health problems including depression and cognitive dysfunction (Sánchez-Villegas et al., 2011). In rats fed a high-saturated fat diet, a reduced H1R binding density in many brain areas was observed (Wu et al., 2013). Interestingly, the reduction of H1R binding densities in some of these areas (substantia nigra and caudate putamen) was prevented by supplementing the HF diet with n-3 polyunsaturated DHA, and also prevented the negative effect of HF in cognitive function. H1R expression is reduced in depressed patients, while omega-3, specifically DHA, levels in serum and red blood cells membranes is reduced in bipolar and major depression patients, with a greater deficit in BD patients (McNamara et al., 2010). In consequence, the evidence suggests that coincident alterations in histaminergic system and lipid composition in depression could be causally linked. Furthermore, histamine clearance (which is essential to avoid excessive histamine activity) is a process dependent on astrocyte reuptake of histamine, which in turn dependents on Na^+/K^+ ATPase activity and is sensitive to ouabain, thus it could have manic like behavioral consequences (Perdan-Pirkmajer et al., 2010; Yoshikawa et al., 2013) (**Figure 2**).

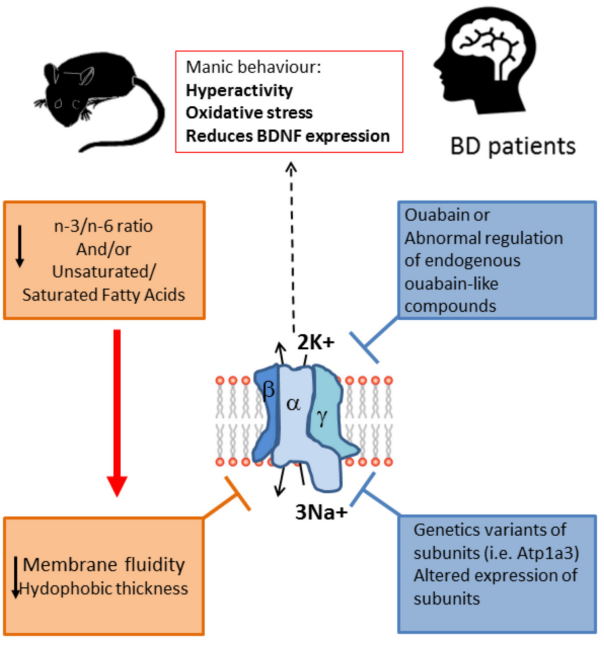

FIGURE 2 | Na^+/K^+ ATPase activity is lower in BD patients and animal models of mania. The enzyme activity is dependent on the fluidity of the membrane, which is lower when the unsaturated/saturated fatty acids ratio at the membrane is decreased, as is the case of BD patients. Moreover, the alteration in the n3/n6 ratio also can modify the membrane fluidity and in turn decrease the Na^+/K^+ ATPase activity. This alteration in lipid composition could be related to the reported lower activity of the enzyme in patients, and it is likely that the lowered activity of the enzyme is related to the symptomatology of BD because reducing the activity of the enzyme pharmacologically with ouabain or by the genetic alteration of the enzyme (Atp1a3 mutant) induces a manic phenotype, with behavioral changes related to mania together with increased oxidative stress and reduction in BDNF expression. Furthermore, some BD patients have been reported to express variants of the enzyme, and also expression of the enzyme is altered in key regions of the limbic system. Altogether the evidence points toward a role for Na^+/K^+ ATPase in the etiology of BD. Unsaturated/saturated fatty acids ratio at the membrane also affects de hydrophobic thickness of the membrane which in turns can affect the pump activity.

MITOCHONDRIA AND BD

Mitochondrial function is critical to neuronal physiology and survival. In spite of its traditional role as the cellular energy generator, mitochondria have other roles (Duchen, 2004). For example, they can decode intracellular signals, in particular, calcium ions (Ca^{2+}) increases (Clapham, 2007). In 2000, Kato presented the mitochondrial dysregulation hypothesis for BD which was suggested by a reduction in phosphocreatine in the frontal lobe of patients with BD observed with magnetic resonance spectroscopy (Kato et al., 1992, 1994). It was shown that BD patients have a lower pH (7.01) in the brain compared to healthy controls (7.05) (Kato et al., 1998). Also, BD patients have higher levels of lactate in cerebrospinal fluid compared to a matching sample of healthy individuals (Regenold et al., 2009) as well as in the brain of bipolar patients, where lactate levels where find to be higher in the anterior cingulate cortex and caudate of BD patients by using 2D proton magnetic resonance spectroscopic imaging (Chu et al., 2013). Lactate is a product of extra-mitochondrial glucose metabolite, usually elevated in resting (no exercising) individuals with mitochondrial dysfunction (Regenold et al., 2009). Furthermore, BD among subjects with mitochondrial diseases is almost 20 times higher (16–21% prevalence) compared to the general population. For example, chronic progressive ophthalmoplegia (CPEO) which is an hereditary mitochondrial disease, sometimes has comorbidity with BD and depression (Suomalainen et al., 1992). Also, Plog 1 (mitochondrial DNA polymerase) is one of the causative genes for CPEO; the transgenic mice (mPolg1 Tg) with a forebrain-specific expression of a mutant Plog 1 gene has characteristics that make it a putative model for BD (Kasahara et al., 2006). Additionally, mitochondria isolated from brains of these transgenic mice have enhanced Ca^{2+} uptake rate (Kubota et al., 2006). Since not all BD patients have Plog 1 mutations, Kubota et al; evaluated candidate genes that were altered both in mPlog1 Tg mice and BD human patients and found that mitochondrial peptidyl-prolyl *cis–trans* isomerase (CypD) gene, was consistently downregulated in mPolg1 Tg mices and BD patients. CypD is a component of the mitochondrial permeability transition pore, using N1M811 an inhibitor of CypD ameliorates the mPolg1 Tg mice behavioral phenotype. CypD sensitizes the brain mitochondria to the transition pore, and its inhibition by CsA or CypD absence improves the complex I-related mitochondrial function and increases mitochondria stability against Ca^{2+} stress (Gainutdinov et al., 2015). In fact, mitochondria of mPolg1 Tg mices have an enhanced Ca^{2+} sequestration rate (Kubota et al., 2006). In their recent review Morris et al., evidenced that mitochondrial function in BD is higher during mania and decreased in depression, and propose that this phasic dysregulation of mitochondrial function is central to BD pathophysiology (Morris et al., 2017). Also, stress and sleep deprivation have been pointed to have a role in BD. These issues could be in part linked to their damaging effect at the level of mitochondrial function (**Figure 3**). Additionally, risks factors associated with BD such as stress and sleep restriction have been reported to induce alterations in mitochondrial function.

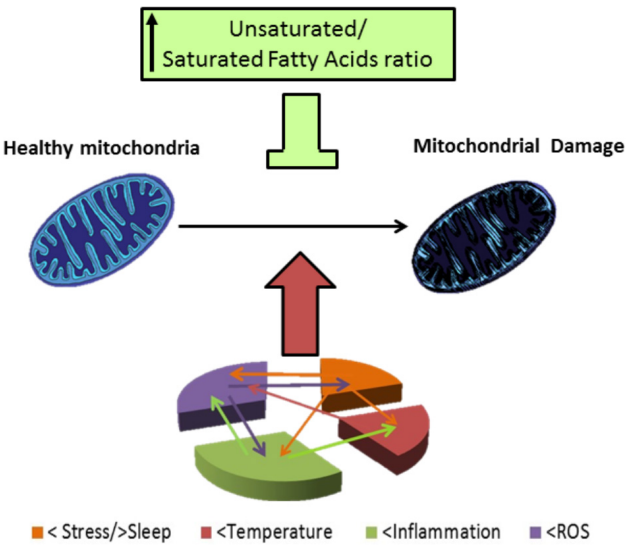

FIGURE 3 | Mitocondrial damage in BD patients. BD patients have altered response to stress and frequently they have a story of life stress events. Also, there are alterations in sleep, mainly reduced sleep at night in BD patients. Stress and poor sleep can induce mitochondrial damage and induce temperature alterations as well as increase inflammation and oxidative stress (level of ROS), temperature has been reported to be increased in BD patients, and high temperature can induce mitochondrial damage. BD patients also have shown to be in a chronic inflammatory state; which; can induce mitochondrial damage, an increased temperature and oxidative stress. ROS are elevated in BD patients and this elevated ROS can worsen the inflammatory state and stress outcome, oxidative stress, and also damage mitochondria. Damaged mitochondria in BD patients have an altered morphology, reduced respiration rates, polymorphisms and deletions in DNA. In consequence of their altered function BD patients have reduced high energy phosphates levels and reduced pH, and their altered mitochondrial function, can in turn increase oxidative stress, neuro-inflammation, and have an impact in stress response. Lipid composition of mithochondrial membrane affect the organelle response to stress, in particular, high levels of PUFAs are protective against different types of stress involved in pathophysiology of BD.

For example, prenatal restraint stress causes a decrease in BDNF mRNA levels, an increase in ROS, and malfunctions in mitochondrial metabolism among other consequences, that can be prevented feeding mothers with a DHA supplemented diet (Feng et al., 2012). Additionally, chronic sleep restriction results in mitochondrial dysfunction in the prefrontal cortex of mice, indicated by morphological alterations and reduction in ATP level (Zhao et al., 2016). Also, chronic sleep deprivation increases oxidative stress, as it increases lipid peroxidation and formation of protein carbonyls as well as oxidation of DNA (Olayaki et al., 2015). Plus, total sleep deprivation or short-term partial sleep deprivation have been shown to induce inflammation (elevated C reactive protein) in healthy adult subjects (Meier-Ewert et al., 2004). However, mitochondrial dysfunction is not only related to BD; it is present in early pathological states in neurodegenerative diseases, as for example in Alzheimer's, Parkinson's, and Huntington's disease, as well as in ischemic stroke, and also in aging brain (Al Shahrani et al., 2017).

Role of PUFAs on Mitochondria Function

Docosahexaenoic acid improved mitochondrial function in animal models of aging and neurodegenerative diseases (Eckert et al., 2013). Animals supplemented with fish oil, which is rich in long-chain n-3 PUFAs, show an increased PUFA content in their mitochondrial membranes. Following these changes in membrane composition, increase in mitochondrial respiration rate and/or Complex IV (cytochrome c oxidase) activity have been observed (McMillin et al., 1992). PUFA supplementation in the diet has also been reported to increase lipid oxidation and reduce energy efficiency in mitochondria, and to reduce oxidative stress, together with an increase in mitochondriogenesis in skeletal muscle of PUFA supplemented rats (Cavaliere et al., 2016). Feeding mices with an n-3 PUFA enriched diet, decrease oxidative stress in cariomiocites, dependent on mitochondrial adaptations, with an increased activity of GR. Therefore, it is clear that PUFAs content in mitochondrial membrane protects the organelle in confronting stress, possibly improving the BD patient's outcome and moreover reducing the risk of developing the disease. Histamine can induce calcium oscillations in mitochondria and increase mitochondrial permeability trough H2 receptor activation (Luo et al., 2013). Chronic stress induces mitochondrial damage (Sonei et al., 2017) and leads to the activation and opening of the mitochondrial transition pore (Kuchukashvili et al., 2012). The neuronal histaminergic system is known to be involved in acute psychological stress (Lkhagvasuren and Oka, 2017) and chronic psychological stress (Ito et al., 1999), histamine levels are increased by stress, therefore, they could be promoting effects of stress in mitochondrial permeability. Plus, histamine released by mast cells during stress is also involved in stress effects in impaired glucose tolerance and reduced sleep acting on H1 receptors (Chikahisa et al., 2017). Accordingly, while PUFAs are a protection factor in the development of BD, Histaminergic system dysregulation, that can be induced by stress and saturated fatty acids in the diet, could participate in generating the damage observed in BD and could underlie behavioral alterations associated to BD symptomatology.

CONCLUSION

Stress, poor sleep, inflammation, and oxidative stress are factors that have long been implicated in neuropsychiatric diseases; they can interact and potentiate each other promoting an environment that alters neuronal physiology. Some of the alterations are related to the neuronal energy machinery focused in the mitochondria and Na^+/K^+ATPase, with consequences in the intracellular Ca^{2+} level and membrane potential. These could particularly be relevant in BD. These molecular and cellular alterations could modify evermore the neuronal circuits that are altered in BD. We propose that altered histaminergic transmission could result from the altered environment produced by stress, poor sleep, inflammation, and oxidative stress. Fatty acid in the diet and consequent fatty acid composition of biological membranes can reduce the damaging effects of this pathoetiological block at both cellular and neurotransmission level. The effects of this neuropsychiatric damage inducing block on the histaminergic transmission system and/or its interaction with fatty acid supplementation are worth exploring in animal models of mania and depression. The H3 autorregulatory system of histaminergic transmission appears to be strongly related to behavioral changes induced by stress, reduced sleep, inflammation, and oxidative stress. Based on our review, we propose that manic state is characterized by high levels of histaminergic transmission while depression is characterized by low levels of histaminergic transmission.

AUTHOR CONTRIBUTIONS

MER wrote the manuscript and did the figures. MAR edited the manuscript and figures.

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REVIEW
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Actions and Mechanisms of Polyunsaturated Fatty Acids on Voltage-Gated Ion Channels

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Polyunsaturated fatty acids (PUFAs) act on most ion channels, thereby having significant physiological and pharmacological effects. In this review we summarize data from numerous PUFAs on voltage-gated ion channels containing one or several voltage-sensor domains, such as voltage-gated sodium (Na_V), potassium (K_V), calcium (Ca_V), and proton (H_V) channels, as well as calcium-activated potassium (K_{Ca}), and transient receptor potential (TRP) channels. Some effects of fatty acids appear to be channel specific, whereas others seem to be more general. Common features for the fatty acids to act on the ion channels are at least two double bonds in *cis* geometry and a charged carboxyl group. In total we identify and label five different sites for the PUFAs. *PUFA site 1*: The intracellular cavity. Binding of PUFA reduces the current, sometimes as a time-dependent block, inducing an apparent inactivation. *PUFA site 2*: The extracellular entrance to the pore. Binding leads to a block of the channel. *PUFA site 3*: The intracellular gate. Binding to this site can bend the gate open and increase the current. *PUFA site 4*: The interface between the extracellular leaflet of the lipid bilayer and the voltage-sensor domain. Binding to this site leads to an opening of the channel via an electrostatic attraction between the negatively charged PUFA and the positively charged voltage sensor. *PUFA site 5*: The interface between the extracellular leaflet of the lipid bilayer and the pore domain. Binding to this site affects slow inactivation. This mapping of functional PUFA sites can form the basis for physiological and pharmacological modifications of voltage-gated ion channels.

Keywords: voltage-gated ion channels, polyunsaturated fatty acids, voltage sensor domain, S4, Excitability disorders

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INTRODUCTION

Fish, fish oils, and polyunsaturated fatty acids (PUFAs; which are major components of fish oils) have beneficial effects on cardiac-, brain-, and muscle-related disorders. This has been shown in a number of studies at different levels:

1. Anthropological studies suggest that the Eskimo and Mediterranean diets, rich in mono- and PUFAs, lower the risk of heart disease and early death (Keys, 1970; Bang et al., 1971) (but see Fodor et al., 2014).

2. Large clinical trials show beneficial effects of dietary fish oil or PUFAs with decreased risk of sudden cardiac death (Burr et al., 1989; de Lorgeril et al., 1994; GISSI-Prevenzione Investigators, 1999; Albert et al., 2002; Marchioli et al., 2002).
3. *In vivo* animal models show that both intraperitoneal and intravenous administration of fish oil or isolated PUFAs prevent induced fatal ventricular arrhythmias (McLennan et al., 1988; McLennan, 1993; Billman et al., 1994, 1997, 1999).
4. *In vitro* models show that PUFAs applied directly to cardiomyocytes terminate arrhythmia and arrhythmia resumes upon removal of PUFAs (Kang and Leaf, 1994).

The last point suggests that PUFAs merely need to partition into the phospholipid cell membrane to exert their antiarrhythmic effect, probably via ion channels, which are responsible for electrical excitability of cells. Despite intense research, the molecular details of the action of PUFAs on ion channels and on excitability are largely unknown. In this review we will summarize what is known about the interaction between PUFAs and one superfamily of ion channels, the voltage-gated ion channels.

Voltage-gated ion channel are pore-forming molecules in the lipid bilayer of most cells, which open in response to alterations in the cell's transmembrane electrical potential (Hille, 2001). Opening of these channels allows the passage of specific types of ion across the cell membrane, thereby initiating and altering essential processes such as, signaling via nervous impulses, or movement via muscle contractions. Ion channels can be regulated by endogenous or exogenous compounds like hormones, pharmaceutical drugs, or toxins. Some compounds, such as PUFAs, can be both endogenous and exogenous.

PUFA effects on ion channels have been reviewed in several excellent papers (Ordway et al., 1991; Meves, 1994; Leaf and Xiao, 2001; Boland and Drzewiecki, 2008) but few, if any, have tried to outline the molecular sites of action and the molecular mechanism of the effects. Even fewer have tried to search for common mechanisms across the channel families. These two aspects are the focus of the present review. We will start with brief overviews of voltage-gated ion channels and of PUFAs. Then, we will summarize the current literature concerning PUFA effects on voltage-gated ion channels. This will be followed by an attempt to explain the data in molecular terms. Finally, we will briefly discuss relevant physiological and therapeutic implications.

THE SUPERFAMILY OF VOLTAGE-GATED ION CHANNELS

The general structure of voltage-gated ion channels has been described in many extensive reviews (e.g., Tombola et al., 2006; Catterall et al., 2007; Bezanilla, 2008; Börjesson and Elinder, 2008). Therefore, we will only briefly describe core features that are pertinent to the subsequent discussion.

The human genome contains 144 genes coding for members of the superfamily of voltage-gated ion channels (http://guidetopharmacology.org/GRAC/ReceptorFamiliesForward?type=IC). **Figure 1** shows an overview of how these 144 channels are classified into families.

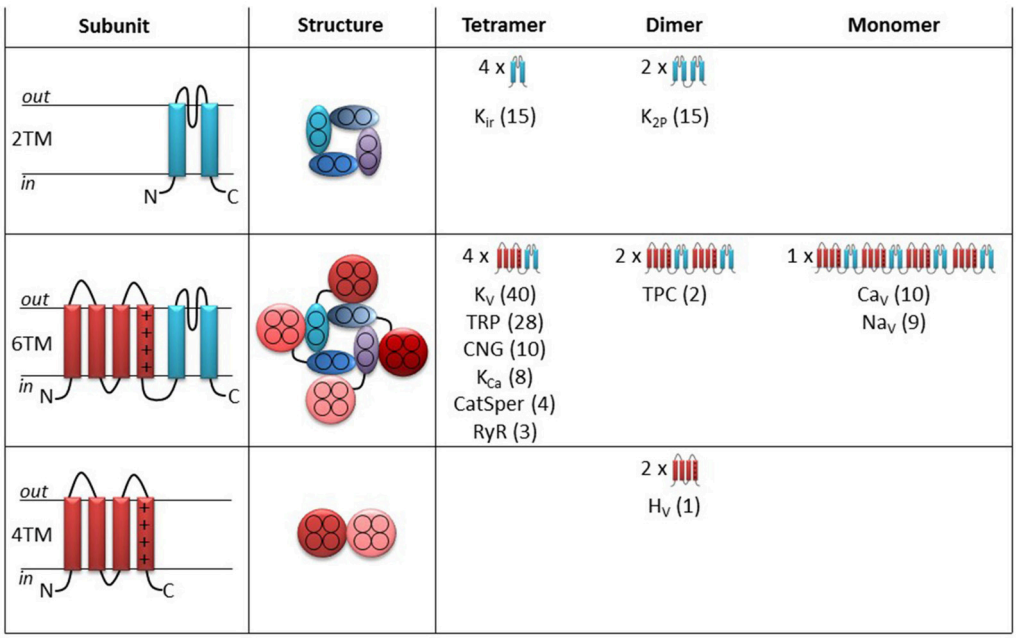

FIGURE 1 | Topology and cartoons on the different ion channels in the superfamily of voltage-gated ion channels. Left column illustrates side view of the topology of a single subunit. Pore forming segments in blue and voltage-sensor domain segments in red. Middle column illustrates top view of the functional ion channel. Right column provides an overview of the different subfamilies and their topology. The numbers in parentheses denote the number of ion channels within each subfamily.

Thirty of the channels (upper row in **Figure 1**) only contain pore-forming subunits (blue in **Figure 1**). Each pore-forming subunit has two transmembrane (2TM) segments with a pore-lining segment in-between (left column in **Figure 1**). Four pore-forming subunits fused together make up a functional channel with a central ion-conducting pore (middle column). This tetrameric structure is referred to as the *pore domain*. The potassium-selective inward rectifiers (K_{ir}) are examples of such channels (**Figure 1**, right column). Also the two-pore potassium (K_{2P}) channels have a similar 3D architecture but are instead formed as dimer-of-dimers (each K_{2P} gene is coding for two linked pore-forming subunits). Channels that contain only the pore domain are not intrinsically voltage sensitive but belong to the superfamily of voltage-gated ion channels because of molecular kinship. These channels are, instead, regulated by mechanical forces or ligands (Kim, 2003; Honoré, 2007).

113 channels in the superfamily of voltage-gated ion channels are composed of pore-forming segments, as described above, linked to voltage sensing segments (red in **Figure 1**) in a six transmembrane (6TM) architecture (**Figure 1**, middle row). These types of channels have a central pore domain surrounded by four *voltage-sensor domains* (VSDs) (**Figure 1**, middle column, middle row). In most cases, the VSD confers voltage dependence to these channels. Molecular details about the voltage-sensing mechanism will be described below when we discuss the molecular mechanism for PUFA action on voltage-gated ion channels. Six families are arranged as tetramers of 6TM subunits (**Figure 1**, right column, middle row): Voltage-gated K (K_V) channels, transient receptor potential (TRP) channels, cyclic nucleotide activated (CNG) channels (including the hyperpolarization and cyclic nucleotide-activated (HCN) channels), calcium-activated K (K_{Ca}) channels, ryanodine receptors (RyR), and cation channels of sperm (CatSper). In contrast, two-pore (TPC) channels are formed as dimers of two linked 6TM subunits, while voltage-gated calcium (Ca_V) and sodium (Na_V) channels are formed as monomers of four linked 6TM subunits.

Finally, one channel, the voltage-gated proton (H_V1) channel is a dimer of 4TM-VSD motifs (**Figure 1**, lower row). This channel lacks the pore domain but allows protons to pass through the center of each VSD (Koch et al., 2008; Tombola et al., 2008).

The present review focuses on PUFA effects on intrinsically voltage-gated ion channels. We will therefore mainly summarize and discuss data from the VSD-containing channels (6TM and 4TM channels in **Figure 1**, the middle and lower rows). Effects on the channels in the upper row will not be covered. However, some of the 2TM channels are highly sensitive to PUFAs, such that some of them have names reflecting regulation by PUFAs. For example, the $K_{2P}4.1$ channel is also referred to as the TWIK-related *arachidonic-acid* activated K (TRAAK) channel. Some of the described PUFA effects on these channels will be briefly mentioned later in this review, when we discuss the molecular mechanism of PUFA effects on intrinsically voltage sensitive ion channels. It should also be noted that some early studies were performed before the molecular identity was known. In these cases we have assigned channels to different families based of their functional characteristics.

CLASSIFICATION AND SOURCES OF FATTY ACIDS

Fatty acids are important messengers in cell signaling and critical components of the phospholipids that constitute the plasma membrane. The general structure of most naturally occurring fatty acids is a carboxylic acid with an unbranched aliphatic hydrocarbon tail. These fatty acids can be classified according to the number of carbon-carbon double bonds in the tail (**Figure 2A**):

- Saturated fatty acids (SFAs) such as stearic acid lack double bonds.
- Monounsaturated fatty acids (MUFAs) such as oleic acid have one double bond.
- Polyunsaturated fatty acids (PUFAs) such as linoleic acid, arachidonic acid (AA), and docosahexaenoic acid (DHA) have two or more double bonds.

A common way to name fatty acids is by the number of carbons and double bonds. For example, DHA is also called 22:6 (22 carbons and six double bonds). Moreover, double bonds can display *cis* geometry (the adjacent carbons are on the same side of the carbon chain) or *trans* geometry (the adjacent carbons are on opposite sides of the carbon chain). *Cis* geometry is most common among naturally occurring unsaturated fatty acids, while *trans* is usually caused by industrial processing of fatty acids (Micha and Mozaffarian, 2009) (**Figure 2A**).

Certain fatty acids, in particular SFAs and MUFAs, can be synthesized *de novo* in the human body (Mullen and Yet, 2015). Others, especially PUFAs, must instead be acquired through the diet (Jakobsson et al., 2006; Kihara, 2012). Dietary intake of α-linolenic acid and linoleic acid (obtained from fish oil or sunflower oil, respectively) is a vital source for PUFAs (**Figure 2B**). The first double bond in α-linolenic acid is located at the third carbon, counting from the methyl end of the tail, and is therefore an n-3 (or ω-3) fatty acid. Linoleic acid, on the other hand, has its first double bond located at the sixth carbon, and is therefore an n-6 (or ω-6) fatty acid. These dietary PUFAs function as precursors in the synthesis of longer PUFAs like the n-3 docosahexaenoic acid (DHA) or the n-6 arachidonic acid (AA) (**Figure 2B**). Non-esterified fatty acids can circulate in the plasma bound to transport proteins such as albumin. These non-esterified free fatty acids are directly available to dissociate from albumin and interact with membrane-bound ion channels (as will be discussed later) or be metabolized by various enzymatic systems (described below).

The phospholipids that constitute the plasma membrane are another important source for fatty acids. Each phospholipid is composed of two fatty acids and a head-group bound to a glycerol backbone (**Figure 3**). SFAs are generally esterified to the first carbon of the glycerol backbone (*sn*1) while PUFAs, or (less commonly) MUFAs, are esterified to the second carbon (*sn*2). The polarity and charge of different phospholipids are determined by the properties of the head group bound to the third carbon of the glycerol backbone (*sn*3). Esterified fatty acids in the plasma membrane can be hydrolyzed to non-esterified free fatty acids, which are then available to interact with ion

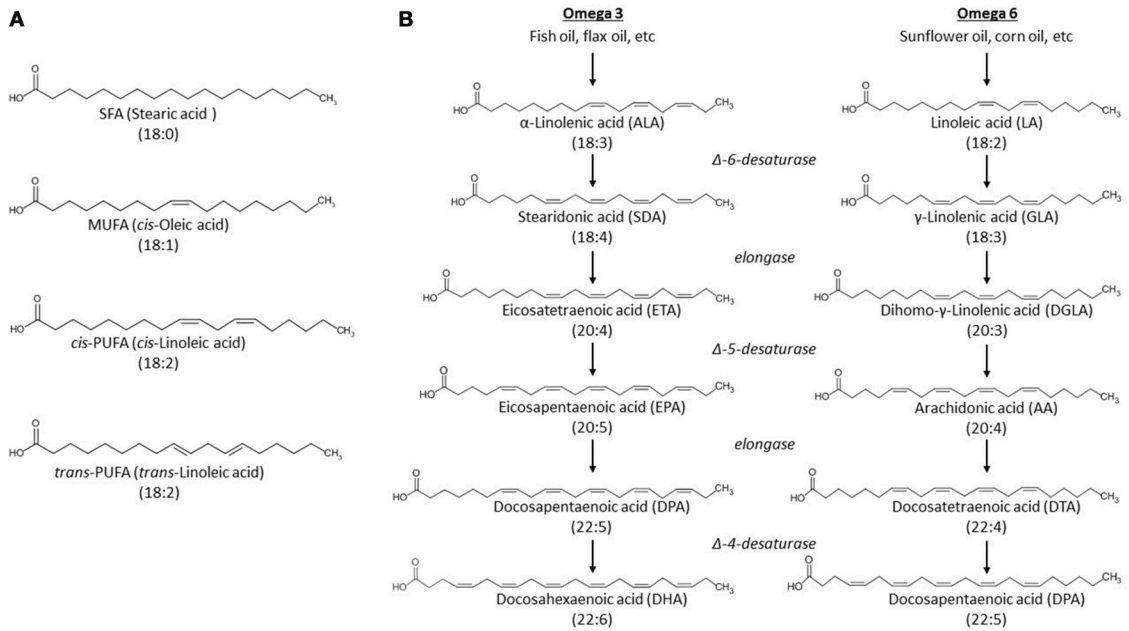

FIGURE 2 | Structures of unesterified fatty acids. (A) Unesterified fatty acids are classified according to the presence, number and geometry of double bonds in the acyl tail. Abbreviations: MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; The PUFA is shown in both *cis* and *trans* geometry. **(B)** Metabolic pathways of n-3 and n-6 fatty acid synthesis. α-linolenic acid and linoleic acid are the precursors of n-3 and 6 PUFAs, respectively. Different desaturases and elongases convert these precursors to different long-chain PUFAs.

channels and other cellular proteins. The hydrolysis of esterified AA has been most extensively studied. It is primarily mediated by four different phospholipases that act at four distinct sites in the phospholipid (**Figure 3**) (Dennis et al., 1991; Siddiqui et al., 2008); Phospholipase A_2 (PLA_2) -mediated hydrolysis of the *sn*2 linkage directly releases AA. In contrast, Phospholipase A_1 (PLA_1), phospholipase C (PLC), or phospholipase D (PLD) -mediated hydrolysis yield precursors of AA (such as 1, 2 diacylglycerol and phosphatidic acid) that require additional enzymatic conversions before non-esterified AA is released. AA and DHA are the most common PUFAs to be found in *sn*2 position in mammalian phospholipids. Release of DHA (or other unsaturated fatty acids) from phospholipids follows the same overall pathway as AA release, although the chemical intermediates formed are different due to differences in the fatty acid acyl tail.

Once released from the plasma membrane, these non-esterified fatty acids may diffuse to and interact with membrane-bound ion channels, take part in intracellular signaling, or be further metabolized by various oxygenases. Metabolism of non-esterified fatty acids is mediated by three main types of oxygenases (**Figure 3**) (Siddiqui et al., 2008; Jenkins et al., 2009): Cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 epoxygenases (CYP). These enzymes produce a family of fatty-acid metabolites named eicosanoids, which includes prostaglandins, leukotrienes, thromboxanes, and epoxides (Siddiqui et al., 2008; Jenkins et al., 2009). Again, the structures of these metabolites depend on the structure of the specific fatty acid that is substrate for oxygenation.

In this review we will focus on the effect of non-esterified PUFAs on voltage-gated ion channels. Several fatty acid metabolites and intermediates formed during phospholipid hydrolysis are also known to modulate the activity of voltage-gated ion channels. However, we will not discuss these interactions here.

EFFECTS OF PUFA ON VOLTAGE-GATED ION CHANNELS

To collect papers describing the effects of PUFA on the VSD-containing channels we searched PubMed for various combinations of voltage-gated ion channels and fatty acids, and extended the list when relevant articles were found during the work. In total we identified, read and analyzed data from 295 original papers containing voltage-clamp data from voltage-gated ion channels published between 1987 and June 2016 (**Table 1**). In addition, we read and analyzed about 400 papers concerning PUFA effects on non-VSD containing channels, review papers, or papers describing PUFA effects on excitability in general.

Historical Notes from 1981–1992

In 1981, Takenaka et al. reported that fatty acids with chain lengths exceeding eight carbons, in the concentration range of 0.2–2.2 mM, decreased the voltage-gated Na current in squid giant axons while leaving the delayed-rectifier K current unaffected. *Cis*-2-decenoic acid, which has ten carbons and a double bond between carbon 2 and 3 was the most effective fatty acid in their experiments (Takenaka et al., 1981). In 1987,

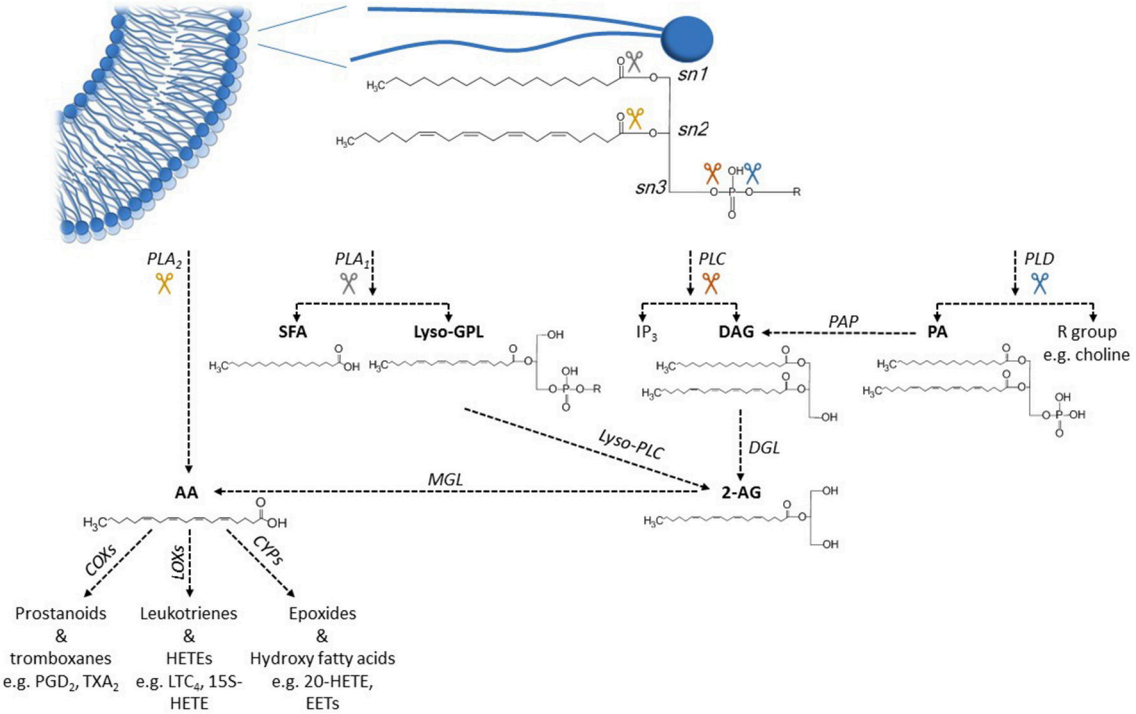

FIGURE 3 | Metabolic pathways of arachidonic acid hydrolysis and oxidation. Phospholipids in the cell membranes commonly have a SFA esterified to *sn*1 position and a PUFA, such as arachidonic acid (AA) esterified to *sn*2 position. Activation of different phospholipases releases AA from phospholipids, either in one enzymatic step (PLA_2) or through several enzymatic steps (PLA_1, PLC, PLD). Unesterified AA can be further metabolized to various eicosanoid metabolites by different COX, LOX, and CYP enzymes. Abbreviations: 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; COX, cyclooxygenase; CYP, cytochrome P450 enzyme; DAG, 1,2-diacylglycerol; DGL, DAG lipase; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatrienoic acid; IP_3, inositol 1,4,5-trisphosphate; LOX, lipooxygenase; LTC_4, leucotriene C_4; Lyso-GPL, lyso-glycerolphospholipid; Lyso-PLC, lysophospholipase C; MGL, monoacylglycerol lipase; PA, phosphatidic acid; PAP, PA phosphatase; PGD_2, prostaglandin D_2; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; SFA, saturated fatty acid; TXA_2, thromboxane A_2.

the same group reported that both saturated and unsaturated medium-chain fatty acids (8–13 carbons) reversibly attenuated voltage-dependent Na currents in squid giant axons by shifting the conductance-vs.-voltage, $G(V)$, curve in a positive direction along the voltage axis (Takenaka et al., 1987). The effect developed much faster upon intracellular application, suggesting an intracellular site of action. The fatty acid concentration needed for 50% reduction of the peak Na current decreased by a factor of 1/3 for each extra carbon. The presence of a carboxyl or hydroxyl group at the ω end of the fatty acid abolished the effect completely. These findings suggested that a hydrophobic interaction between the fatty acid and Na channel could be an important factor for the effect.

Longer chain fatty acids like palmitic acid (16:0), linoleic acid (18:2), and linolenic acid (18:3) decreased both Na and K currents, but the effects were irreversible, probably because of high concentrations tested would result in micelle formation. Finally, in 1988, by using α-cyclodextrin to dissolve the fatty acids, this group reported that long-chain PUFAs produced effects similar to medium-chain fatty acids (Takenaka et al., 1988). Intracellularly applied AA (20:4) reversibly suppressed the Na current of the squid giant axon with little effect on the K current. 180 μM AA reduced the Na current by 50%, which is a concentration almost ten times lower than required for the medium-chain fatty acid, 2-decenoic acid. Longer PUFAs, Docosatetraenoic (22:4) and DHA (22:6), had effects quantitatively similar to AA. Shorter PUFAs, linoleic acid (18:2) and linolenic acid (18:3), had smaller effects than AA, while the effects of the MUFA oleic acid (18:1) were even smaller, and the SFA stearic acid (18:0) had almost no effect.

In 1989, Bregetovski et al. reported that 2-decenoic acid increased the open probability of K_{Ca} channels up to 10-fold in the membrane of smooth muscle cells from the human aorta (Bregestovski et al., 1989). They suggested that 2-decenoic acid alters the Ca^{2+}-binding mechanism of the channel. The same year Linden and Routtenberg reported that low concentrations (1–50 μM) of the MUFA oleic acid (18:1), the PUFAs linoleic acid (18:2), and linolenic acid (18:3), but not the SFA stearic acid (18:0) or the trans-isomer of oleic acid blocked the Na current in N1E-115 neuroblastoma cells (Linden and Routtenberg, 1989); 5 μM oleic acid decreased the peak Na current by 36%. K currents were not affected while both T-type and L-type Ca currents were blocked. This study also excluded the possible explanation that

TABLE 1 | List of general effects and references to all articles analyzed in the present review.

Family	Amplitude	G(V)	ss-inact.	Inactivation	No articles	References
K_V1-4	↓	←	←	Faster	76	a
K_V7	↑	←	–	–	9	b
K_V10-12	↑↓	←	–	–	5	c
K_{Ca}	↑	←	–	–	53	d
TRP	↑	–	–	–	24	e
CNG	↑↓	–	–	–	2	f
RyR	↑↓	–	–	–	3	g
Catsper/TCP	↑↓	–	–	–	4	h
Na_V	↓	↔	←	–	41	i
Ca_V	↓	←	←	Faster	69	j
H_V	↑	←	–	–	8	k

The arrows denote the general effect in each family. Double arrows denote mixed effects. A dash denote that the parameter has not been investigated, there is no effect, or that it is not applicable. a, (Takenaka et al., 1987, 1988; Premkumar et al., 1990; Rouzaire-Dubois et al., 1991; Damron et al., 1993; Villarroel, 1993; Chesnoy-Marchais and Fritsch, 1994; Honoré et al., 1994; Lee et al., 1994; Lynch and Voss, 1994; Gubitosi-Klug et al., 1995; Poling et al., 1995; Nagano et al., 1995a; Poling et al., 1996; Soliven and Wang, 1995; Wang and Lu, 1995; Nagano et al., 1997; Garratt et al., 1996; Smirnov and Aaronson, 1996; Villarroel and Schwarz, 1996; Gilbertson et al., 1997; Horimoto et al., 1997; Keros and McBain, 1997; Bogdanov et al., 1998; Bringmann et al., 1998; Devor and Frizzell, 1998; Dryer et al., 1998; Hatton and Peers, 1998; Visentin and Levi, 1998; Bittner and Müller, 1999; Colbert and Pan, 1999; Singleton et al., 1999; Yu et al., 1999; Casavant et al., 2000; Wilson et al., 2000; Holmqvist et al., 2001; Kehl, 2001; McKay and Jennings, 2001; Takahira et al., 2001; Erichsen et al., 2002; Müller and Bittner, 2002; Ramakers and Storm, 2002; Seebungkert and Lynch, 2002; Xiao et al., 2002; Danthi et al., 2003; Ferroni et al., 2003; Judé et al., 2003; Fioretti et al., 2004; Oliver et al., 2004; Sokolowski et al., 2004; Angelova and Müller, 2006, 2009; Feng et al., 2006; Kang et al., 2006; Jacobson et al., 2007; Szekely et al., 2007; Zhao et al., 2007; Börjesson et al., 2008, 2010; Guizy et al., 2008; Xu et al., 2008; Zhang M. et al., 2008; Boland et al., 2009; Koshida et al., 2009; Li et al., 2009; Wang et al., 2009; Decher et al., 2010; Börjesson and Elinder, 2011; Lai et al., 2011; Kong et al., 2012; Heler et al., 2013; Carta et al., 2014; Ottosson et al., 2014; Bai et al., 2015; Farag et al., 2016; Yazdi et al., 2016). b, (Béhé et al., 1992; Villarroel, 1993, 1994; Yu, 1995; Doolan et al., 2002; Milberg et al., 2011; Liin et al., 2015, 2016a,b; Moreno et al., 2015). c, (Schledermann et al., 2001; Liu and Wu, 2003; Wang et al., 2004; Guizy et al., 2005; Gavrilova-Ruch et al., 2007). d, (Bregestovski et al., 1989; Kirber et al., 1992; Ling et al., 1992; Ahn et al., 1994; Duerson et al., 1996; Zou et al., 1996; Twitchell et al., 1997; Devor and Frizzell, 1998; Stockand et al., 1998; Denson et al., 1999, 2000, 2005, 2006; Barlow et al., 2000; Wu et al., 2000; Fukao et al., 2001; Lu et al., 2001, 2005; Zhang et al., 2001; Zhang P. et al., 2008; Clarke et al., 2002, 2003; Lauterbach et al., 2002; Li et al., 2002, 2010; Ye et al., 2002; Hamilton et al., 2003; Gauthier et al., 2004, 2014; Zheng et al., 2005, 2008; Yang M. et al., 2005; Sun et al., 2007, 2009; Morin et al., 2007a,b,c; Gebremedhin et al., 2008; Godlewski et al., 2009; Lai et al., 2009; Wang et al., 2011a,b; Enyeart and Enyeart, 2013; Harris et al., 2013; Latorre and Contreras, 2013; Hoshi et al., 2013a,b,c,d; Kacik et al., 2014; Martín et al., 2014; Olszewska et al., 2014; Yan et al., 2014). e, (Chyb et al., 1999; Watanabe et al., 2003; Kahn-Kirby et al., 2004; Hu et al., 2006; Jörs et al., 2006; Oike et al., 2006; Reiter et al., 2006; Andersson et al., 2007; Hartmannsgruber et al., 2007; Matta et al., 2007; Vriens et al., 2007; Rock et al., 2008; Delgado and Bacigalupo, 2009; Shimizu et al., 2009; Parnas et al., 2009a,b; Zhang et al., 2010; Bavencoffe et al., 2011; Motter and Ahern, 2012; Shah et al., 2012; Sukumar et al., 2012; Zheng et al., 2013; Redmond et al., 2014; Ruparel et al., 2015). f, (Fogle et al., 2007; Verkerk et al., 2009). g, (Honen et al., 2003; Woolcott et al., 2006; Muslikhov et al., 2014). h, (Mochizuki-Oda et al., 1993; Asano et al., 1997; Liu et al., 2006; Gutla et al., 2012). i, (Linden and Routtenberg, 1989; Wieland et al., 1992, 1996; Fraser et al., 1993; Charpentier et al., 1995; Kang et al., 1995, 1997; Xiao et al., 1995, 1998, 2000, 2001, 2004, 2005, 2006; Kang and Leaf, 1996; Vreugdenhil et al., 1996; Bendahhou et al., 1997; Fyfe et al., 1997; Macleod et al., 1998; Lee et al., 1999, 2002; Leifert et al., 1999; Ding et al., 2000; Harrell and Stimers, 2002; Leaf et al., 2002; Hong et al., 2004; Jo et al., 2005; Kim et al., 2005; Isbilen et al., 2006; Pignier et al., 2007; Duan et al., 2008; Dujardin et al., 2008; Gu et al., 2009, 2015; Nakajima et al., 2009, 2010; Fang et al., 2011; Guo et al., 2012; Wolkowicz et al., 2014; Safrany-Fark et al., 2015; Wannous et al., 2015). j, (Keyser and Alger, 1990; Finkel et al., 1992; Hallaq et al., 1992; Huang et al., 1992; Shimada and Somlyo, 1992; Damron and Bond, 1993; Dettbarn and Palade, 1993; Pepe et al., 1994; Törnquist et al., 1994; Williams et al., 1994; Roudbaraki et al., 1995; Nagano et al., 1995b; Schmitt and Meves, 1995; van der Zee et al., 1995; Petit-Jacques and Hartzell, 1996; Shimasue et al., 1996; Shuttleworth, 1996; Uehara et al., 1996; Unno et al., 1996; Damron and Summers, 1997; Munaron et al., 1997; Striggow and Ehrlich, 1997; Xiao et al., 1997; Hazama et al., 1998; Chen et al., 1999, 2001; Fang et al., 1999; Liu and Rittenhouse, 2000, 2003; Vellani et al., 2000; Zhang et al., 2000; Barrett et al., 2001; Bringmann et al., 2001; Fiorio Pla and Munaron, 2001; Hirafuji et al., 2001; Krutetskaia et al., 2001; Liu et al., 2001, 2004, 2008, 2015; Luo et al., 2001; Mignen and Shuttleworth, 2001; Ferrier et al., 2002; Soldati et al., 2002; Swan et al., 2003; Yagami et al., 2003; Guermouche et al., 2004; Guibert et al., 2004; Oz et al., 2004; Talavera et al., 2004; Danthi et al., 2005; Erriquez et al., 2005; Rychkov et al., 2005; Yang K. T. et al., 2005; Chemin et al., 2007; Holmes et al., 2007; Liu, 2007; Feng et al., 2008; Rimmerman et al., 2008; Barbara et al., 2009; Heneghan et al., 2009; Mitra-Ganguli et al., 2009; Roberts-Crowley and Rittenhouse, 2009, 2015; Rocha and Bendhack, 2009; DeCostanzo et al., 2010; Cazade et al., 2014; Cui et al., 2014; Thompson et al., 2014). k, (DeCoursey and Cherny, 1993; Kapus et al., 1994; Gordienko et al., 1996; Lowenthal and Levy, 1999; Hourton-Cabassa et al., 2002; Morgan et al., 2002, 2007; Kawanabe and Okamura, 2016).

fatty acid effects were produced by increased fluidization of the membrane.

In 1991, Rouzaire-Dubois et al. showed that several MUFAs and PUFAs induced or accelerated inactivation of K_V channels via a direct mechanism (not activation of protein kinase C). For instance, 5 μM of oleic acid accelerated the inactivation by a factor of about 10. Among the 18-carbon fatty acids, linoleic acid (18:2) was the most potent inactivator (50-fold acceleration at 5 μM), followed by oleic acid (18:1), linolenic acid (18:3), elaidic acid (18:1, trans), and stearic acid (18:0) which did not affect the inactivation time course at all.

In 1992 several papers on different ion channels established that low μM concentrations of PUFAs affect voltage-gated ion channels, opening as well as closing (Béhé et al., 1992; Finkel et al., 1992; Hallaq et al., 1992; Huang et al., 1992; Kirber et al., 1992; Ling et al., 1992; Shimada and Somlyo, 1992; Wieland et al., 1992).

At about the same time several influential studies were published suggesting that PUFA or PUFA-metabolites had direct effects on other, non-voltage-gated, ion channels (Buttner et al., 1989; Giaume et al., 1989; Kim and Clapham, 1989; Kurachi et al., 1989; Ordway et al., 1989; Anderson and Welsh, 1990; Cantiello et al., 1990; Hwang et al., 1990; Kim and Duff, 1990).

General Effects

Despite the multiple different types of ion channels and PUFAs included in this review, the effects PUFAs have on voltage-gated ion channels are surprisingly general and can be summarized

in a few points (**Table 1**). However, it should be noted that quantitative differences do exist.

i. Alteration in voltage dependence of ion channels: A common finding is that PUFAs shift the $G(V)$ and/or the steady-state inactivation curves in a negative direction along the voltage axis (**Figures 4A,B**). Such a shift of the $G(V)$ curve opens the channel, while this shift of the steady-state inactivation curve closes (inactivates) the channel. For Na_V and Ca_V channels, shifts of the steady-state inactivation curve tend to be larger than shifts of the $G(V)$ curves. As a consequence, Na_V and Ca_V channels are generally inhibited by PUFAs. In contrast, K_V channels which in many cases are less affected by steady-state inactivation at resting voltage are typically activated by PUFAs.

ii. Alteration in maximal conductance of ion channels: PUFAs are also able to increase or decrease the conductance at positive voltages (either open probability or the single-channel conductance), where the conductance is not affected as a consequence of the $G(V)$ shift (**Figure 4C**). In many cases, there is a combination of effect i and ii (**Figure 4D**). Despite these combined effects it is relatively easy to distinguish them without curve fitting. Increased conductance can be measured directly at voltages where the conductance has saturated while a $G(V)$ shift can be measured at the foot of the curve (e.g. at 10% of maximal conductance in control–the error for the $G(V)$ curve shown in **Figure 4D** is only 1.7 mV if the maximal conductance is increased by 50%).

iii. Alteration in the time course of ion channel kinetics: Consistent with the negative shift of the channel's voltage dependence in negative direction along the voltage axis, the opening kinetics are sometimes faster (**Figure 4E**) and the closing kinetics slower (**Figure 4F**) in the presence of PUFAs. There are also multiple reports of a PUFA-induced acceleration of channel inactivation (**Figure 4G**).

Specific Effects—Family by Family

Although many of the PUFA effects are general for the voltage-gated ion channels, there are quantitative and qualitative differences. We will therefore briefly describe the specific PUFA effects on different sub-families of voltage-gated ion channels. **Table 1** describes the general effects for the specific families and lists the references.

K_V Channels

The largest and most studied family when it comes to PUFA effects on voltage-gated ion channels is the family of voltage-gated K (K_V) channels. Because of the size and diversity of this family, we will divide this family into three groups of subfamilies. Subfamilies that are not included in our description below have not, to our knowledge, been studied with respect to PUFAs.

K_V1–4: K_V channels within these subfamilies open rapidly and thereby cause fairly fast repolarization of the action potential. Therefore, these channels have special importance for neurons that fire with high frequency. Some of these channels [such as

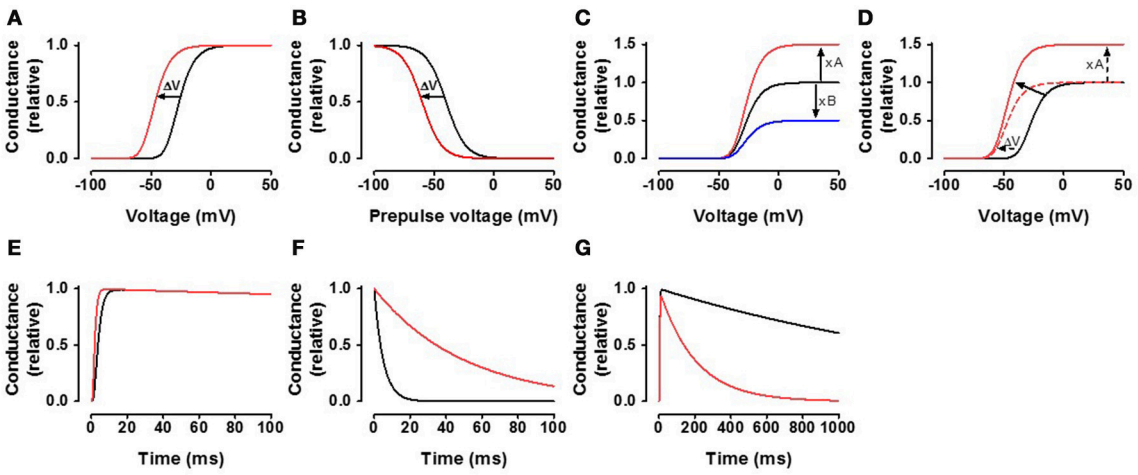

FIGURE 4 | General effects of the fatty acids on the channels. (A) The black curve represents a typical control conductance-vs.-voltage curve [$G(V) = 1/(1+\exp((V-V_{½})/s))^n$, where V is the membrane voltage, $s = 8$ mV, $V_{½} = -40$ mV, $n = 4$] for a voltage-gated ion channel. The red curve is the control curve shifted by −20 mV. **(B)** The black curve represents a typical steady-state inactivation curve [$G(V_{PP}) = 1/(1+\exp((V-V_{½})/s))$, $s = -8$ mV, $V_{½} = -40$ mV]. The red curve is the control curve shifted by −20 mV. **(C)** The black curve represents a typical control curve as in **(A)**. The red curve is the control curve increased by a factor 1.5. The blue curve is the control curve decreased by multiplying by 0.5. **(D)** The black curve represents a typical control curve as in **(A)**. The red continuous curve is an example where the curve is both shifted in negative direction along the voltage axis and increased. The amplitude increase can reliably be measured at high voltages where the conductance levels out. The shift can reliably be measured at the foot of the conductance curve (at 10% of the max value of the control curve) without normalization of the curve. The shift of the curve is −20 mV. Measured at the foot, when the maximum conductance is increased by 50%, the shift is over-estimated by 1.7 mV (–21.7 mV instead of –20 mV). **(E)** The black curve represents a typical activation time course ($\tau = 2$ ms, $n = 4$, $\tau_{inact} = 2$ s). The red curve is a two fold increase in opening rate. **(F)** The black curve represents a typical single exponential channel closure ($\tau = 5$ ms). The red curve is 10 times slower. **(G)** The black curve represents a typical channel inactivation ($\tau = 2$ s), while the red inactivates 10 times faster.

K_V4 channels which generate transient outward (I_{to}) neuronal and cardiac K currents] also inactivate rapidly and are thus sometimes referred to as A-type K_V channels. Other members within this subfamily, such as Kv2.1, inactivate slowly generating persistent K currents, in the physiological time frame. Some studies describe PUFA-induced increases in native K currents of unclear molecular identity (e.g., Horimoto et al., 1997; Ferroni et al., 2003; Fioretti et al., 2004), however the most commonly observed PUFA effect on fast native K currents (Lynch and Voss, 1994) and heterologously expressed K_V1–4 channels is inhibition (by 20–100% at ∼10 μM PUFA). This inhibition is commonly associated with an acceleration of the time course of channel inactivation. PUFA effects on channel voltage dependence are less consistent, but the most commonly described are negative voltage shifts of $G(V)$ and/or steady-state inactivation curves. The overall effect is typically a reduced current, but a few exceptions describe PUFA-induced activation of K_V1–4 channels (Zhao et al., 2007; Börjesson et al., 2008, 2010; Zhang M. et al., 2008; Börjesson and Elinder, 2011).

K_V7: K_V channels within this subfamily open slowly and are referred to as slow delayed rectifiers. K_V7 channels underlie the neuronal M current, which contributes to the negative resting membrane potential in neurons, and the cardiac I_{Ks} current, which contributes to the repolarization in cardiomyocytes. PUFAs are reported to activate both natively and heterologously expressed K_V7 channels. PUFA-induced increases of K_V7 current amplitudes are associated with a small negative shift in the $G(V)$ curve (roughly −5 to −10 mV by 10 μM PUFA). There are, however, some inconsistencies concerning the role of the auxiliary subunit KCNE1 during PUFA exposure. The cardiac I_{Ks} channel is a complex between $K_V7.1$ and KCNE1. Doolan et al. find that PUFA effects on the I_{Ks} channel require the presence of KCNE1 (Doolan et al., 2002). In contrast, we describe that KCNE1 causes reduced PUFA sensitivity of the I_{Ks} channel compared to $K_V7.1$ alone (Liin et al., 2015). Moreover, Moreno *et al.* show that PUFA effects on the I_{Ks} channel vary over time (Moreno et al., 2015).

K_V10–12: These subfamilies contain the $K_V10.1$ channel (= EAG1) and the $K_V11.1$ channel (= hERG or ERG1). $K_V11.1$ forms the major portion of the rapid delayed rectifier current (I_{Kr}), which is critical in correctly timing the repolarization of cardiac action potentials. Mutations in $K_V11.1$ and compounds targeting I_{Kr} channels can cause long QT syndrome and subsequent lethal ventricular fibrillation. Most PUFA studies on this group have been performed on the $K_V11.1$ channel, with a single study performed on $K_V10.1$. The effects in this small group are mixed. Both current reductions and current increases have been reported. The $G(V)$ curve is negatively shifted in most studies. This shift is rather large for $K_V10.1$, around −30 mV at 10 μM for all PUFAs studied (Gavrilova-Ruch et al., 2007). Several studies also suggest that PUFAs speed up closure (inactivation) of these channels.

K_{Ca} Channels

The family of Ca-activated K channels contains three types of channels: Big, intermediate, and small conductance channels. Only the $K_{Ca}1.1$ (BK) family is clearly voltage dependent as it is opened by alterations in membrane voltage *in addition to* increases in the intracellular Ca^{2+} concentration. Almost all studies of PUFA effects on K_{Ca} channels have been performed on $K_{Ca}1.1$ channel. This channel is essential for the regulation of smooth muscle tone and neuronal excitability. PUFAs, even at submicromolar concentrations, increase the maximum conductance *and* shift the $G(V)$ curve in negative direction along the voltage axis. In addition, the $K_{Ca}1.1$ channel is quite sensitive to PUFA metabolites (Meves, 2008). Recent studies have mapped the binding site for PUFAs to a region near the intracellular gate (Hoshi et al., 2013d; Tian et al., 2016).

TRP Channels

The transient receptor potential (TRP) channels form a large family, consisting of 28 channels divided in six subfamilies. TRP channels are for example involved in mediating the sensations of cold, heat, and pain. These channels are fairly non-selective and therefore conduct several types of cations (e.g., Na^+, Ca^{2+}). TRP channels are generally described as being activated by PUFAs. However, many of these studies measured TRP channel activity indirectly using fluorescence-based calcium imaging, which provides limited information about TRP channel voltage dependence and the time course of TRP currents. In studies that include electrophysiological recordings (primarily from TRPVs, TRPCs, TRPAs, and *drosophila* TRPs), the amplitude of TRP currents are found to increase many-fold following application of >10 μM PUFA. Moreover, Shimizu et al. describe a PUFA-induced negative shift in the $G(V)$ curve of TRPP3 channels (Shimizu et al., 2009). However, TRPM channels are an exception among TRP channels, as they are almost completely inhibited by PUFAs (Andersson et al., 2007; Parnas et al., 2009b; Bavencoffe et al., 2011).

Na_V Channels

The family of voltage-gated Na channels contains the first ion channel to be discovered and explored electrophysiologically (Hodgkin and Huxley, 1952a,b), and later, cloned and sequenced (Noda et al., 1984). Na_V channels generate action potentials in neurons, the heart, and other muscles. Thus, they are important targets for the regulation of excitability. With few exceptions, PUFAs reduce Na_V currents. However, PUFAs also shift the $G(V)$ and steady-state inactivation curves of most Na_V channels in a negative direction along the voltage axis. In general, the steady-state inactivation curve is shifted more than the $G(V)$ curve. These shifts have conflicting results; the *G(V)*-curve shift opens channels and thereby increase excitability, while the steady-state inactivation curve shift inactivates/closes channels and thereby decrease excitability. Altogether, these mixed effects result in reduced excitability.

Ca_V Channels

Voltage-gated Ca channels have two critical functions: Generating (or boosting) action potentials, and conducting extracellular Ca^{2+} ions into the cell where they can act as a second messenger. PUFA effects on Ca_V channels have been studied rather extensively. The effects are very similar to the effects on Na_V channels, that is, the maximal conductance is decreased, and $G(V)$ and steady-state inactivation curves are shifted in a negative direction along the voltage axis, with

the steady-state inactivation shift being larger than the $G(V)$ shift. In addition, the inactivation time course is in some cases accelerated. Altogether, these mixed effects result in reduced excitability.

H_V Channels

The proton channel, which was cloned only 10 years ago (Ramsey et al., 2006; Sasaki et al., 2006), deviates from all other ion channels in lacking the conventional ion-conducting pore domain. However, the voltage sensing mechanism is similar to the other voltage-gated ion channels; the difference is that two VSDs act together as a dimer (Koch et al., 2008; Lee et al., 2008). The effects of PUFAs on the H_V channels are reminiscent of the effects on the other channels, suggesting that at least some of the effects are conferred by the VSD. PUFAs increase the maximal current of H_V channels–for most other channels the maximal current is decreased. The shift of the $G(V)$ is in the negative direction along the voltage axis, but the size is smaller than for most other channels. One surprising finding is that the PUFA carboxyl charge is not important for this effect (Kawanabe and Okamura, 2016).

Other Voltage-Gated Ion Channels

Several other ion channels belonging to the superfamily of voltage-gated ion channels have been explored with respect to PUFA effects, but many of them are difficult to study in biophysical detail. For several of the families only few studies have been performed, often with mixed data, making it difficult to draw general conclusions. These families are briefly mentioned here and the references are found in **Table 1**. The family of cyclic-nucleotide gated (CNG) ion channels contains two types of channels–hyperpolarization-activated cyclic nucleotide gated (HCN) channels, which are highly voltage dependent (even though the polarity is opposite to most other ion channels), and the non-voltage dependent CNG channels. HCN channel have an important role as pacemaker channels in the sino-atrial node of the heart. AA has been found to directly facilitate HCN channel opening, and rats fed a diet enriched with fish oil show reduced pacemaker currents and consequently reduced heart rate (see **Table 1**). The ryanodine receptor (RyR) family is an intracellular cation channel critical for the regulation of intracellular levels of Ca^{2+}. PUFAs have been reported both to increase and decrease the RyR current. CatSper channels and TCP channels are molecularly related. CatSper channels are found in the plasma membrane of sperm while TCP channels are found in intracellular endolysosomes. Here the effects of PUFAs are also mixed.

SITES AND MECHANISMS OF ACTIONS OF PUFA

There are some general properties of fatty acids that are often described as being required to induce the PUFA effects described above (e.g., Xiao et al., 1997, 1998; Danthi et al., 2003, 2005; Börjesson et al., 2008; Liin et al., 2015):

(a) At least two double bonds in the acyl tail are required. Therefore, PUFAs induce these effects while SFAs and MUFAs generally do not. However, there is usually no clear difference between n-3 and n-6 PUFAs. Also, there is no large or systematic difference between PUFAs with respect to chain lengths from 16 to 24 carbons.
(b) *Cis*-geometry of the double bonds in the acyl tail is required. *Trans*-geometry renders the PUFAs ineffective.
(c) The negative charge of the carboxyl group is required. Uncharged methyl esters of PUFAs generally lack effects.

In addition, PUFAs need to remain in their intact form. Experiments conducted with non-metabolizable PUFA analogs (such as ETYA) and cyclooxygenase inhibitors (that prevent PUFA metabolism) show that the PUFAs themselves, and not their metabolites, induce these general effects. Some exceptions, however, have been reported (Twitchell et al., 1997; Lee et al., 2002; Judé et al., 2003).

Despite the large number of studies published (**Table 1**), only a few PUFA sites of action have been described and little has been described concerning the mechanism by which PUFAs interact with voltage-gated ion channels.

The first major question is whether the reported effects of PUFAs on the voltage-gated ions channels are direct channel effects or if they are mediated via non-specific membrane effects. In general, the concentrations needed for the PUFA effects are relatively low (1–10 μM), ruling out unspecific membrane fluidizing effects (Pound et al., 2001). Moreover, there is no correlation between a PUFA's propensity to fluidize the membrane and their effects on voltage-gated ion channels (Villarroel and Schwarz, 1996). Alterations of the lipid membrane by soaking out cholesterol affect ion channel function but do not affect acute PUFA effects (Moreno et al., 2015). Further, the onset and washout of the effect on K_V channels is very rapid (2–3 s), suggesting a direct channel effect (Poling et al., 1996). An early suggestion that PUFAs may bind directly to voltage-gated ion channels came from experiments on Na_V channels in which the PUFA eicosapentaenoic acid (EPA) inhibited the binding of a radio-labeled toxin to cardiac Na_V channels (Kang et al., 1995; Kang and Leaf, 1996). Further evidence that PUFAs have direct ion channel effects is provided by the demonstration that single point mutations in various voltage-gated ion channels also affects the ability of PUFAs to modulate those channels (e.g., Xiao et al., 2001; Börjesson and Elinder, 2011; Ottosson et al., 2014; Liin et al., 2015).

Secondly, we may ask on which side of the membrane the PUFAs act. Whereas, most studies have used extracellular application of PUFAs, one study made a direct comparison of PUFA-induced effects upon PUFA application from either side of the membrane. They found no difference in PUFA effects on K_V channels based on the side of application (Oliver et al., 2004). In contrast, some studies have demonstrated ion channel modulation when PUFAs are applied extracellularly but fail to observe modulation when PUFAs are added intracellularly (Honoré et al., 1994; Poling et al., 1995, 1996; Garratt et al., 1996; Kehl, 2001; McKay and Jennings, 2001;

Guizy et al., 2008). Yet other studies primarily observe effects when the PUFAs are applied to the intracellular side (Boland et al., 2009; Decher et al., 2010). These differences in the side of action may be explained by differences in the predominant PUFA sites of action in different types of ion channels.

Five Sites of Action

From our analysis of PUFA publications in the field we have identified five sites of actions (**Figures 5A,B**). The first two sites are located in the ion-conducting pore, one at the intracellular entrance (PUFA site 1), and the other at the extracellular entrance (PUFA site 2). The third is located at the VSD-to-pore domain linker close to the intracellular gate (PUFA site 3). The last two are located at the interface between the extracellular part of the ion channel and the outer leaflet of the lipid bilayer from which PUFAs electrostatically interact with the VSD (PUFA site 4) or the pore domain (PUFA site 5).

PUFA Site 1–the Intracellular Cavity

Several studies have identified the intracellular part of the pore lining S6, with residues facing the intracellular cavity, as critical for the PUFA effects. A common mechanism is an open-channel block causing a time dependent current reduction–an inactivation.

A single point mutation of domain I of the cardiac $Na_V1.5$ channel (N406K) clearly reduces the inhibitory effect of DHA (Xiao et al., 2001). The negative shift of the steady-state inactivation curve is also attenuated. The identified amino-acid residue is located in the middle of S6, facing the intracellular cavity, in a similar position where local anesthetics bind to domain IV of a rat brain Na_V channel (Ragsdale et al., 1994). However, the molecular detail why the steady-state inactivation curve is shifted by DHA has not been described.

In $K_V1.1$ channels, DHA and AA, but also the uncharged anandamide induces inactivation by interacting with hydrophobic residues lining the inner cavity of the pore

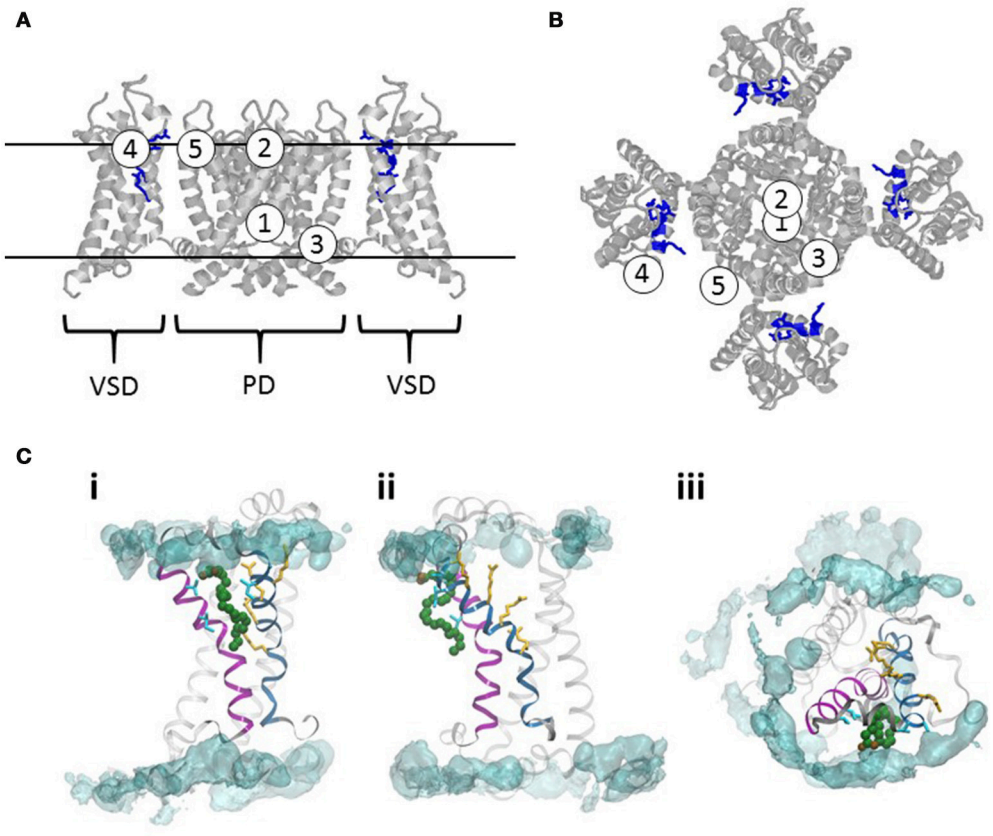

FIGURE 5 | Sites of actions of PUFAs on voltage-gated ion channels. (A) A homology model of the Shaker K_V channel based on the structure of the $K_V2.1/1.2$ chimera (Long et al., 2007; Henrion et al., 2012). Side view. VSD denote one voltage-sensor domain. PD denotes the pore domain. For clarity, the VSD in the front and the back are removed. The long loop between S3 and S4 are removed (residues 337–353). The two continuous lines delineate the approximate outer and inner borders of the lipid bilayer. The **Figures 1**–**5** denote five proposed sites of actions of PUFA. **(B)** Top view of the channel in **(A)**. **(C)** Interaction site for a DHA molecule with the VSD of the Shaker K_V channel. The helix in magenta is S3 and the helix in blue is S4. The four yellow amino acid residues are the four gating charges [R362 (in the top), R365, R368, and R371]. The four residues in cyan (two in S3, residues 325 and 329; two in S4, 359 and 360) are the residues identified to be close to the PUFA binding site (Börjesson and Elinder, 2011). A typical binding pose for a DHA molecule in green is from Yazdi et al. (2016). The POPC lipid bilayer is represented by a cyan iso-density surface corresponding to the positions of lipid nitrogens in the simulation at 5% occupancy. The left and middle panels are the VSD viewed along the membrane from two different angles. The right panel is the VSD viewed from the extracellular side.

(Decher et al., 2010). The inactivation was suggested to be caused by open-channel block by PUFA binding to the cavity of the channel. $K_V1.5$ has been proposed to be inactivated via a similar mechanism. Point mutations combined with computer docking support PUFA binding in the cavity (Bai et al., 2015).

In the Ca-activated $K_{Ca}3.1$ (= SK4 or I_{K1}) channel, which is not voltage sensitive despite having VSDs, AA inhibits the current. This inhibition is completely prevented by the T250S mutation at the inner end of the pore loop, together with the V275A mutation in the middle of S6, close to residue 250 (Hamilton et al., 2003). Furthermore, introducing the threonine and the valine in the equivalent positions of the AA-insensitive $K_{Ca}2.2$ (= SK2) channel makes this channel sensitive to AA. Thus, AA interacts with the pore-lining amino acids of $K_{Ca}3.1$ to inhibit the channel.

Thus, several studies on different ion channels have identified the middle of S6, in the cavity, as a major determinant for PUFA interactions.

Another type of channel-inactivating pore-interacting mechanism has been described for AA on $K_V3.1$ (Oliver et al., 2004). AA is equally effective from either side of the membrane. AA-induced inactivation was not affected by the presence of TEA at the extracellular or intracellular side of the channel protein. These results rule out open-channel block as the mechanism underlying AA-induced inactivation, but suggest a lipid-induced closure of the "pore gate".

PUFA Site 2–the Extracellular Entrance of the Ion Conducting Pore

$K_V1.1$ (Garratt et al., 1996), $K_V1.2$ (Garratt et al., 1996; Poling et al., 1996), $K_V1.5$ (Honoré et al., 1994; Bai et al., 2015), and $K_V3.1a$ (Poling et al., 1996) are inactivated by PUFAs via a proposed open-channel block where the pore is accessed from the extracellular side. Point mutations combined with computer-guided docking support a PUFA binding site at the extracellular entrance of the pore (Bai et al., 2015).

PUFA Site 3–the Intracellular Gate (Lower End of S6 and S4—S5 Linker)

Some studies have identified a PUFA site at the inner end of S6 or in the S4–S5 linker, which are close to each other and form the intracellular gate of the channel (Long et al., 2005). In the absence of detailed data we have brought them together to a single site. The difference from PUFA site 1 and 2 is that this site is outside the central axis of the channel and that this site thus can host PUFA molecules to open the channel by bending the gate open.

The Ca^{2+}-activated $K_{Ca}1.1$ (= BK) channel is, in contrast to the $Na_V1.5$ and the $K_{Ca}3.1$ channels described above, opened by several PUFAs such as DHA, AA and α-linolenic acid. Hoshi and collaborators have identified Y318 near the cytoplasmic end of S6 in the $K_{Ca}1.1$ channel as a critical determinant of the stimulatory action of DHA (Hoshi et al., 2013d; Tian et al., 2016). The Y318S mutation greatly diminishes the channel's response to DHA, but not to AA or α-linolenic acid.

$K_V4.2$ inactivates very quickly upon application of AA, while the inactivation of the Shaker K_V channel is fairly unaffected. Transplanting the Shaker S4–S5 linker to $K_V4.2$ attenuates the effect of AA on the $K_V4.2$ channel, and conversely, transplanting the $K_V4.2$ S4–S5 linker to the Shaker K_V channel makes the Shaker K_V channel more sensitive to AA (Villarroel and Schwarz, 1996). Molecular docking approaches using a $K_V4.2$ homology model predicted a membrane-embedded binding pocket for AA comprised of the S4–S5 linker on one subunit and several hydrophobic residues within S3, S5, and S6 from an adjacent subunit (Heler et al., 2013). The pocket is conserved among K_V4 channels.

Pufa Site 4–Lipoelectric Effects on S4 Charges of the Voltage-Sensor Domain

It is well-known that the lipid environment is important for the function of voltage-gated ion channels. Crystal structures show that phospholipids are making close and specific contacts with the channel (Long et al., 2007). Molecular dynamics simulations suggest that the negatively charged phosphate group of phospholipids make electrostatic interactions with the positive charges of the voltage sensor (Freites et al., 2005; Sansom et al., 2005). Experiments altering the charge of the phospholipids show that the charge of the phospholipids is necessary for proper function of voltage-gated ion channels (Schmidt et al., 2006). Free PUFA molecules can also affect ion-channel gating. PUFA molecules in the extracellular solution can quickly incorporate in the extracellular leaflet of the phospholipid bilayer; the hydrophobic tail is tucked into the hydrophobic part of the bilayer and the carboxyl group is facing the extracellular water (Feller et al., 2002; Yazdi et al., 2016). The PUFA molecules are most likely everywhere in the lipid bilayer but they could potentially be clustered around ion channels (Yazdi et al., 2016).

In studies of the Shaker K_V channel and several K_V7 channels we have identified a site between the extracellular leaflet of the lipid bilayer and S4 of the VSD. Mutational analysis and molecular dynamics simulations have suggested that the PUFA molecules interact between the transmembrane segments S3 and S4 and the lipid bilayer (**Figure 5C**) (Börjesson and Elinder, 2011; Yazdi et al., 2016). The electric charge of free PUFA molecules in the lipid bilayer affects the gating machinery of the VSD (Börjesson et al., 2008, 2010; Börjesson and Elinder, 2011; Ottosson et al., 2014; Liin et al., 2015, 2016b; Yazdi et al., 2016). Because lipophilicity and electrostatic forces are central in this model, we have called this the *lipoelectric* mechanism.

Pufa Site 5–Lipoelectric Effects on the Pore Domain

PUFAs modulate the $K_V1.4$ channel inactivation. It has been suggested that the PUFA molecule partition in the membrane as has been suggested for PUFA site 4. The difference is that the negatively charged PUFA molecule line up outside the pore domain and from this position the acidic head group of the PUFAs raises the pK_a of H508 in the pore domain. This raised pK_a of the histidine reduces the K^+ occupancy of the selectivity filter, stabilizing the C-type inactivated state (Farag et al., 2016).

Helical Screw and a Mechanism by Which PUFAs Can Open An Ion Channel

Of all five sites described above, the mechanism by which PUFAs affect K_V channels via PUFA site 4 has been studied in most detail. In the remaining part of this section we will focus on this PUFA mechanism. The mechanism by which voltage-gated ion channels sense membrane voltage is central for this effect (reviewed for instance in Armstrong, 1981; Keynes and Elinder, 1999; Bezanilla, 2000; Swartz, 2004; Börjesson and Elinder, 2008). Therefore, we will here, in brief, describe the mechanism for voltage sensing.

The four VSDs connected to a central ion-conducting pore domain make, in most cases, the channel voltage sensitive. Each VSD has four transmembrane segments labeled S1 to S4. The fourth transmembrane segment, S4, has several positively charged amino-acid residues (blue sticks in **Figure 6**) interspaced by two hydrophobic residues. The transmembrane segments S1 to S3 host negative counter charges (red sticks in **Figure 6**) that neutralize the positive S4 charges in the transmembrane section of the VSD. The positive charges of S4 can change partners and thereby slide along the rest of the VSD (from the deepest state C4 to the open state O in **Figure 6**). At negative membrane voltages, S4 is close to the intracellular side (the down state) and at positive membrane voltages S4 is close to the extracellular side (the up state) of the membrane. At resting states C4 and C3 most S4 charges are below the hydrophobic barrier (Tao et al., 2010) (the green phenylalanine in **Figure 6**). Upon activation three to four charges of each S4 move across the barrier, in three to four discrete steps. The total movement is around 13 Å, even though distances from 7 to 15 Å have been reported (e.g., Ruta et al., 2005; Campos et al., 2007; Delemotte et al., 2011; Henrion et al., 2012).

S4 not only slides along S1–S3 during activation but also rotates around its longitudinal axis because the positive charges are spiraling around S4 (**Figure 6**). This means that the top positive charge in S4 (R1) moves in a spiral from the center of the channel to the extracellular surface and then along the surface (arrows in **Figure 6**). Thus, fixed negative charges at or close to the extracellular surface of the channel can electrostatically "pull" S4 to open the channel, while fixed positive charges could do the opposite. For instance, charged residues in the extracellular linkers connecting the transmembrane segments of a voltage-gated ion channel can control the voltage dependence of the channel (Elinder et al., 2016).

Our data are consistent with one (or several) PUFA molecules interacting with the VSD close to a cleft between the extracellular ends of S3 and S4 (Börjesson and Elinder, 2011). Experimental data from the Shaker K_V channel suggests that it is mainly the C1 → O transition that is affected by the PUFA molecules and that the top charge of S4, which moves horizontally along the lipid bilayer during this last step, is the most important charge for the effect.

Data Supporting the Lipoelectric Model

Here we list experimental support for the proposed lipoelectric model. Most of the experiments have been performed on the Shaker K_V channel. Some experiments have also been performed on $K_V7.1$ and $K_V7.2/3$ channels:

(1) The sign and size of the PUFA charge is critical for the effect. (i) A PUFA molecule, expected to be at least partially negatively charged at neutral pH, increases the current (**Figure 7A**, red curve) by shifting the $G(V)$ curve in negative direction along the voltage axis (**Figure 7B**, red curve), as expected from an electrostatic mechanism. (ii) If the PUFA molecule is not permanently charged at neutral pH, alterations in pH are expected to affect the PUFA effect. In fact, pH has a pronounced effect on the $G(V)$ shift for PUFAs (**Figure 7C**, red symbols). At pH 6.5 there is no shift as if the PUFA molecule is uncharged. At pH 9 or 10 the shift is saturated as if the PUFA molecule is fully negatively charged. The midpoint value of the curve is at pH 7.9 for the Shaker K channel. This surprisingly high value compared to the predicted pK_a value in solution of pH 4.9 suggests that the local pH at the surface is radically different from the bulk solution. Similar effects have been described for $K_V7.1$ ($pK_a = 7.7$) and $K_V7.2/3$ ($pK_a = 7.5$). Alteration of the charge of amino acids close to the binding site can alter

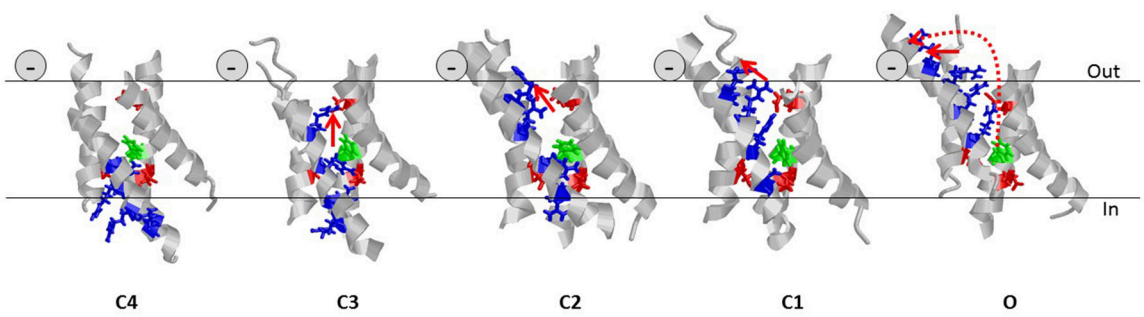

FIGURE 6 | Helical screw and the lipoelectric effect. Five states of the VSD of the Shaker K_V channel are shown (Henrion et al., 2012). For clarity, only the transmembrane segments (224–246, 278–300, 311–332, 354–377) are shown and the intra- and extracellular loops are removed. Gating charges (residues R362, R365, R368, and R371) of S4 are shown as blue sticks. Negative counter charges (E283, E293, and D316) are shown as red sticks. The hydrophobic barrier in S2 is shown in green (F290). The continuous red arrows indicated the movement of the charge of R362 in each step. The dotted red arrow in state O denotes the complete movement of R362, from state C4 to state O. The negative sign denotes the position of the carboxyl group of the PUFA molecule.

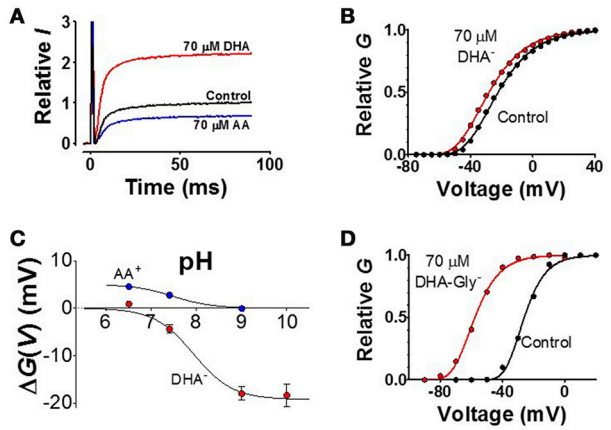

FIGURE 7 | Examples of experimental data. (A) K current though Shaker K_V channels at a voltage step to V = −40 from a holding voltage of −80 mV. The negatively charged DHA increases the current (red curve), while the positively charged arachidonoyl amine (AA^+) decreases the current (Börjesson et al., 2010). **(B)** The normalized conductance-vs.- voltage curve for control and 70 µM DHA (Börjesson et al., 2010). **(C)** pH dependent shift of the *G*(*V*) curves (Börjesson et al., 2008, 2010). **(D)** Decreasing the pK_a value of the DHA molecule, and thereby charging the molecule, by adding a glycine motif increases the shift (Liin et al., 2015) (data on the $K_V7.1$ channel).

the apparent pK_a value of PUFAs (Börjesson and Elinder, 2011). Interestingly the auxiliary subunit KCNE1 alters the pK_a value of $K_V7.1$ to $pK_a = 8.6$ to render the channel essentially insensitive to PUFA at neutral pH (Liin et al., 2015). (iii) If the charge is essential, an uncharged molecule should not shift the *G*(*V*) and a permanently charged should shift the *G*(*V*) as much the PUFA molecule at high pH. In fact, uncharged methyl esters of the PUFAs do not shift the *G*(*V*) despite competing with PUFAs for the same site (Liin et al., 2015). Designed PUFAs with a shifted pK_a value, for instance docosahexaenoyl glycine (DHA-Gly), shifts the *G*(*V*) much more than a PUFA molecule at neutral pH (**Figure 7D**) (Liin et al., 2015, 2016b). Most importantly, a positively charged "PUFA" should shift the *G*(*V*) in positive direction along the voltage axis and reduce the current. This is in fact the case (**Figures 7A,C** blue trace and symbols) (Börjesson et al., 2010; Liin et al., 2015). Also these positively charged PUFA analogs show pH dependence, but now the effect is in opposite direction (**Figure 7C**).

(2) The positions and valence of the charges on S4 are critical. To investigate the PUFA interaction with S4, in closer detail, we decorated the extracellular end of S4 in the Shaker K_V channel with positively charged residues in different positions (Ottosson et al., 2014). The major findings were the following: (i) moving the top charge R1 in S4 from position 362 to 359 (by constructing the A359R/R362Q mutant) increased the effect by DHA by a factor of about two (**Figure 8A**). (ii) Adding more arginines than just one sometimes increased the effect; adding two extra charges (356R and 359R) to the existing top charge of S4 (R362) increased the PUFA-induced *G*(*V*) shift by a factor of three (**Figure 8A**). Because there are three (positively charged) arginines in the sequence 356–362 in this construct, we have called this the 3R channel. (iii) A positively charged residue on the opposite side to R359 (the most influential charge) of the α-helical S4 (i.e., R361) abolished the PUFA-induced *G*(*V*) shift (**Figure 8B**), supporting the idea that S4 rotates and that R1 is moved along the bilayer surface (at least in its last step). (iv) Negatively charged residues introduced at these specific positions in S4 had opposite effects to positive charges supporting electrostatic effects.

(3) PUFA mainly act on the final channel-opening step. A voltage-gated ion channel undergoes several voltage dependent transitions between closed (C) states before it enters into the open (O) state (**Figure 8C**). PUFAs act on the voltage-sensor transitions and can theoretically act on any of the transitions. It is possible to differentiate effects on the early voltage-dependent transitions, before the channel reaches the open state, and the final voltage-dependent transition, which open the channel, in the Shaker K_V channel by introducing a set of mutations in S4 (the ILT mutation) (Smith-Maxwell et al., 1998). We found that DHA only has a minor effect on the early transitions, and that almost all effects of DHA are on the last step (Börjesson and Elinder, 2011). This means that the critical, PUFA-sensitive step, is when R1 moves from a position close to the pore domain to a position close to the lipid bilayer (C1 to O in **Figure 6**). In $K_V7.1$ channels, both early S4 movements and S4 movements associated with channel opening are affected by PUFA (Liin et al., 2016b). However, the relative PUFA effect on these different gating transitions in the $K_V7.1$ channel remains to be quantified.

Other Properties Important for the Effect of PUFAs

The lipolelectric mechanism described above clearly explains why the charge of the PUFA molecule plays such an important role. However, we have less information about why multiple double bonds in *cis* geometry in the tail are required, and we have no information about the length of the tail. Double bonds restrict the conformational freedom in C=C bonds of the fatty acid but double bonds in *cis* geometry (**Figure 2**), causes the chain to bend and explore conformations not found for saturated fatty acids–the more *cis* double bonds the more curved the molecule is. The curvedness goes from a kink for one double bond to hairpin shapes for five or six double bonds. DHA, a PUFA with a 22-carbon chain and six *cis* double bonds, undergoes fast conformational changes and has a highly flexible structure (Eldho et al., 2003). In contrast, double bonds in *trans* geometry (**Figure 2**) do not cause the chain to bend much, having a shape similar to straight saturated fatty acids. Thus, it is not surprising that saturated fatty acids and *trans* PUFAs lack effects on ion channels if a curved shape is required.

The same pattern for the effective molecules is not restricted to voltage-gated ion channels but also to fatty-acid activation of K_{2P} channels where fatty acid-induced stimulation requires at least one C=C bond and the anionic (COO-) form of the fatty

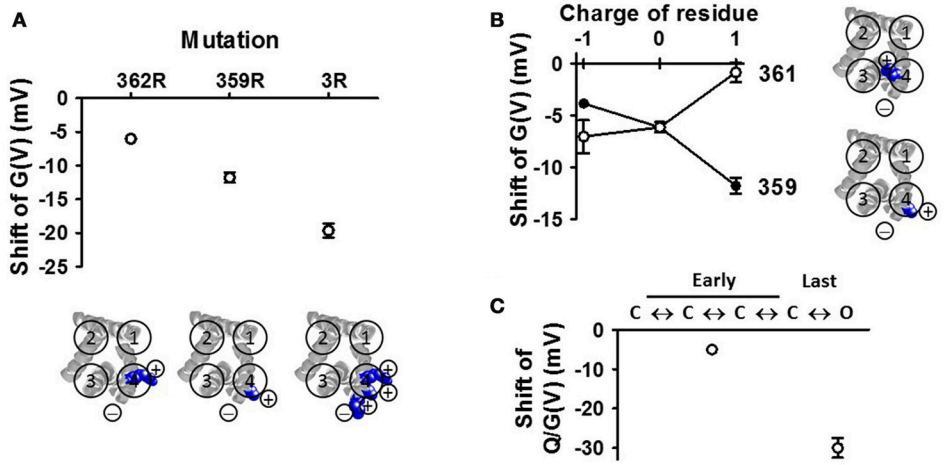

FIGURE 8 | VSD mutations affect the DHA effect on the Shaker K_V channel (A) The number and positions of the arginines in the top of S4 affects the DHA-induced *G*(*V*) shift (Ottosson et al., 2014). The 3R channel contains the positive charges R356, R359, and R362. Only the transmembrane parts are shown (residues 224–246, 278–300, 311–332, 354–377) **(B)** Altering the valence or altering the side of S4 for the charge alters the effect of DHA (Ottosson et al., 2014). **(C)** Effects on the early and late transitions are measured on the conducting and non-conducting ILT-mutant (Börjesson and Elinder, 2011).

acid (Lotshaw, 2007). Unesterified DHA molecules are predicted to infiltrate certain spaces between the transmembrane helices of rhodopsin (Grossfield et al., 2006). The dynamic changes in the protein during gating would thus be influenced by the packing of DHA within these spaces, which could explain how DHA facilitates conformational changes in rhodopsin upon activation (Feller and Gawrisch, 2005). This flexibility can explain the promiscuity of the PUFAs, why they act on so many channels and sites.

PHYSIOLOGICAL AND THERAPEUTIC CONCENTRATIONS

The concentration of unesterified PUFAs available to affect voltage-gated ion channels in different tissues is largely unknown. It is therefore difficult to assess the physiological relevance of the PUFA effects described in this paper. A concentration range of 1–30 μM of PUFA is often effective for experimental modulation of voltage-gated ion channels. The concentration of unesterified PUFA in plasma has been reported to be roughly 10–50 μM (Burtis and Ashwood, 1998; De Caterina et al., 2000; Fraser et al., 2003; Siddiqui et al., 2008). This plasma concentration of PUFA can dramatically increase to 130–400 μM during consumption of certain diets (Kuriki et al., 2002; Fraser et al., 2003; Siddiqui et al., 2008). Moreover, the local PUFA concentration in specific tissues may be increased during pathological conditions such as ischemia and epileptic seizures (Hochachka, 1986; Siesjö et al., 1989). Based on these reported PUFA concentrations it seems plausible that most voltage-gated ion channels that are PUFA sensitive would experience some degree of PUFA modulation under physiological as well as pathological conditions. The outcome of different synergistic and opposing PUFA modulations in terms of, for instance, neuronal and cardiac excitability is hard to predict and would depend on the PUFA sensitivity and relative importance of each type of ion channel.

CONCLUDING REMARKS

In the present review, we have suggested five different PUFA-binding sites. Two of the sites (PUFA site 1 and PUFA site 2) are located in the ion conducting pore and binding to these sites reduce the current. Two of the sites (PUFA site 3 and PUFA site 4) can either increase or decrease the open probability of the channel by either affecting the gate (PUFA site 3) or the voltage sensor (PUFA site 4). Finally, one site in the periphery of the pore domain (PUFA site 5) can regulate slow inactivation by acting on distance. We suggest that all five sites can exist in a single ion channel and the overall effect is determined by the relative contributions of the five sites.

AUTHOR CONTRIBUTIONS

FE and SL designed the study, analyzed data, and wrote the manuscript.

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Inhibition of Intermediate-Conductance Calcium-Activated K Channel (KCa3.1) and Fibroblast Mitogenesis by α-Linolenic Acid and Alterations of Channel Expression in the Lysosomal Storage Disorders, Fabry Disease, and Niemann Pick C

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The calcium/calmodulin-gated KCa3.1 channel regulates normal and abnormal mitogenesis by controlling K^+-efflux, cell volume, and membrane hyperpolarization-driven calcium-entry. Recent studies suggest modulation of KCa3.1 by omega-3 fatty acids as negative modulators and impaired KCa3.1 functions in the inherited lysosomal storage disorder (LSD), Fabry disease (FD). In the first part of present study, we characterize KCa3.1 in murine and human fibroblasts and test the impact of omega-3 fatty acids on fibroblast proliferation. In the second, we study whether KCa3.1 is altered in the LSDs, FD, and Niemann-Pick disease type C (NPC). Our patch-clamp and mRNA-expression studies on murine and human fibroblasts show functional expression of KCa3.1. K_{Ca} currents display the typical pharmacological fingerprint of KCa3.1: Ca^{2+}-activation, potentiation by the positive-gating modulators, SKA-31 and SKA-121, and inhibition by TRAM-34, Senicapoc (ICA-17043), and the negative-gating modulator, 13b. Considering modulation by omega-3 fatty acids we found that α-linolenic acid (α-LA) and docosahexanenoic acid (DHA) inhibit KCa3.1 currents and strongly reduce fibroblast growth. The α-LA-rich linseed oil and γ-LA-rich borage oil at 0.5% produce channel inhibition while α-LA/γ-LA-low oils has no anti-proliferative effect. Concerning KCa3.1 in LSD, mRNA expression studies, and patch-clamp on primary fibroblasts from FD and NPC patients reveal lower KCa3.1-gene expression and membrane expression than

in control fibroblasts. In conclusion, the omega-3 fatty acid, α-LA, and α-LA/γ-LA-rich plant oils, inhibit fibroblast KCa3.1 channels and mitogenesis. Reduced fibroblast KCa3.1 functions are a feature and possible biomarker of cell dysfunction in FD and NPC and supports the concept that biased lipid metabolism is capable of negatively modulating KCa3.1 expression.

Keywords: KCa3.1/SK4 channel, linolenic acids, fabry disease, fibroblasts, ion channel pharmacology

INTRODUCTION

The intermediate-conductance calcium/calmodulin-gated potassium channel KCa3.1 (encoded by the *KCNN4* gene) is expressed in a variety of tissues such red and white blood cell lineage, epithelia, and endothelia (Wei et al., 2005). Notably, induction of KCa3.1 is suspected to drive abnormal cell proliferation, inflammation, and pathological organ remodeling (fibrosis) in heart, kidneys, lungs, some cancers, and atherosclerosis and neo-angiogenesis (for review see: Roach et al., 2013; Feske et al., 2015; Huang et al., 2015; Köhler et al., 2016). Accordingly, pharmacological modulation of the channel is considered a way to treat disease in humans. Hence, several small molecule blockers and positive and negative-gating modulators are available and Icagen compound, Senicapoc, was found clinically safe (for recent review see: Christophersen and Wulff, 2015). Also the endogenous omega-6 fatty, arachidonic acid (AA) and AA-metabolites such as some 11,12 epoxyeicosatrienoic acid and 20-hydroxyeicosatetraenoic acid (20-HETE), have been found to inhibit cloned human KCa3.1 that required mechanistically the inner pore lining amino acid residues, T250 and V275 as putative interaction sites (Hamilton et al., 2003; Kacik et al., 2014). However, the cell biological meaning of this modulation of KCa3.1 by omega-3/6 fatty acid is unclear.

Interestingly, KCa3.1-functions have been suggested to be compromised in Fabry disease (FD; Choi et al., 2014, 2015; Choi and Park, 2016), a X-linked lysosomal storage disorder (LSD), in which defective lysosomal targeting of mutated α-galactosidase A encoded by the *GLA* gene causes globotriaosylceramide (Gb3) accumulation. Mechanistically, Gb3 has been suggested to downregulate KCa3.1-expression, fibroblast growth, differentiation into myofibroblasts, and collagen expression. These impairments could be reversed by channel activation (Choi et al., 2015).

In keeping with these previous findings, we characterized in the first part of this study KCa3.1 functions in a murine fibroblast cell line and human dermal fibroblasts and tested inhibitory actions of α-linolenic acid (α-LA) and docosahexaenoic acid (DHA) on channel functions and fibroblast mitogenesis.

In the second part, we tested whether disease-related impairments of fibroblast KCa3.1 are features of the two LSDs, FD and Niemann-Pick disease type C (NPC).

In this study, we found that murine and human dermal fibroblasts express amounts of functional KCa3.1 channels. We show strong sensitivity of fibroblast mitogenesis to α-LA and DHA.

KCa3.1 gene expression and KCa3.1 currents were reduced in two different LSDs.

METHODS

Cells and Patients

Fibroblast Cultures

Murine 3T3-L1 fibroblasts were cultured in DMEM supplemented with 10% FCS (Biochrom KG, Berlin, Germany). Primary human dermal fibroblast cultures were made from punch biopsies from FD and NPC patients as well as from a healthy donor (according to the guidelines of the local Ethics Committee, permit no. PI16/0227). Fibroblasts from patients' skin biopsies were cultured in DMEM supplemented with 10% FCS (Biochrom KG, Berlin, Germany) and cells were used at passages 1–4. For patch-clamp experiments, cells were seeded on coverslips and used within 24 h.

This study was carried out in accordance with the recommendations of the local Ethics Committee (CEICA) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the local Ethics Committee (CEICA).

Patients

The FD patients included in this study displayed the classic FD phenotypes: A 32-years old male (p.Y216fs*15 hemizygous) showed acroparesthesias, hypohidrosis, and mild proteinuria and renal glycolipid deposits and frequent gastrointestinal disturbances. The 56-years old female (p.G183V heterozygous) showed despite her heterozygous genotype clear and typical clinical symptoms like acroparesthesias, hypohidrosis, mild proteinuria, and renal glycolipid deposits, cardiac arrhythmias, and frequent gastrointestinal disturbances. An atypical 50-years old female FD patient harbored a complex intronic haplotype (CIH) (c.-10C > T, c.369 + 990C > A, c.370-81_370-77delCAGCC, c.640-16A > G, c.1000-22C > T, heterozygous) (Gervas-Arruga et al., 2015) and showed ischemic coronary artery disease with repetitive balloon catheter inventions, stent implantations, and valve insufficiency. A 41-years old male FD patient with a complex intronic haplotype (CIH) (c.-10C > T, c.369 + 990C > A, c.370-81_370-77delCAGCC, c.640-16A > G, c.1000-22C > T, hemizygous; Gervas-Arruga et al., 2015) who had a different phenotype with mild renal deposits in podocytes and normal glomerular function, but showed severe acroparesthesias and two transitory episodes of ischemic stroke.

The Fabry patients started enzymatic replacement therapy in 2011 with agalsidase alfa weekly and are clinically stable at present.

The 31-years old male NPC patient (*NPC1* p.Q775P heterozygous) did not show neurological manifestation yet.

The patient developed severe spleen enlargement with extreme invasion of foam cells and was splenectomized.

Patch-Clamp Electrophysiology

In brief, Ca^{2+}-activated K^+ currents were measured in the whole-cell configuration using an EPC10-USB amplifier (HEKA, Electronics, Lambrecht-Pfalz, Germany) and a K pipette solution (intracellular) containing 1 μM Ca^{2+} free (in mM): 140 KCl, 1 $MgCl_2$, 2 EGTA, 1.71 $CaCl_2$ (1 μM $[Ca^{2+}]_{free}$), and 5 HEPES (adjusted to pH 7.2 with KOH). The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 10 glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH). For maximal KCa3.1-activation we applied SKA-31 or SKA-121 at a concentration of 1 μM that is ∼10-times the concentration for half-maximal activation.

For data acquisition and analysis we used the patch-master program (HEKA). Ohmic leak currents of up to 1 nS were subtracted where appropriate. We quantified outward currents at a 0 mV holding potential (to avoid contamination with non-selective cation currents and chloride currents). Capacitance values (a measure of cell surface and thus cell size) were significantly higher in fibroblasts from female and male FD GLA Intronic hemizygous than in Ctrl (Ctrl, 38 ± 5 pF; ♀ FD *GLA* Intronic heterozygous, 80 ± 12 pF ($P < 0.05$ vs. Ctrl, Student's *T*-Tests); ♂ FD *GLA* Intronic hemizygous, 107 ± 10 pF ($P < 0.05$ vs. Ctrl, Student's *T*-Tests); ♀ FD *GLA* p.G183V heterozygous, 24 ± 5 pF; ♂ FD *GLA* p.Y216fs*15 hemizygous, 50 ± 8 pF, $n = 6$; ♂ *NPC1* p.Q775P heterozygous, 34 ± 6 pF, $n = 5$).

Gene Expression Studies

RNA Isolation and Reverse Transcription

Total RNA was isolated with TriReagent (Sigma, Saint Louis, Missouri, USA) following manufacturer's protocol, and further purified using RNA Clean-up and Concentration-Micro-Elute kit (Norgen Biotek, Thorold, Canada). Genomic DNA digested using the Ambion DNA-free kit (Invitrogen, Carlsbad, California, USA). Quantity and purity of extracted RNA were determined by spectrophotometry (NanoDrop 1000, Thermofisher, Waltham, MA) and stored at −80°C for later use. Integrity of RNA samples and successful digestion of genomic DNA were ensured by gel electrophoresis. Reverse transcription was performed with 600 ng of total RNA by using the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, California, USA) and random hexamers following the manufacturer's protocol.

Quantitative RT-PCR

cDNA obtained from 20 ng of total RNA was amplified in triplicates using the SYBR Select Master Mix and a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, California, USA) using the following cycle protocol: 95°C, 15 s and 60°C, 60 s repeated for 40 cycles. As final step, a melting curve analysis was carried out to verify correct amplification. The following primers were used: KCa3.1-F: 5′-CATCACATTCCT GACCATCG-3′; KCa3.1-R: 5′-ACGTGCTTCTCTGCCTTGTT-3′, GAPDH-F: 5′-GGGATCAATGACCCCTTCAT-3′; GAPDH-R: 5′-GCCATGGAATTTGCCAT-3.

Data were analyzed with LinRegPCR software (Ruijter et al., 2009) and gene expression levels relative to GAPDH expression as reference gene and normalized to control were calculated using the formula:

$$\%\ of\ \ Control = \frac{Efficiency(KCa3.1)^{Cq(Control)-Cq(Sample)}}{Efficiency(GAPDH)^{Cq(Control)-Cq(Sample)}}$$

Proliferation Assay

Measurements of cell proliferation were done using the vital colorimetric Janus-Green assay, with some modifications as described previously in more detail (Olivan-Viguera et al., 2013). In brief, 3T3-L1 fibroblasts were seeded at the same density (80,000 cells/well) in 12-well-plates (flat bottom, Costar, Corning Inc. NY, USA) and cultured in DMEM medium supplemented with L-glutamine, with 10% fetal bovine serum and 1% penicillin/streptomycin, in the presence of DHA (10 μM), α-LA (10–500 μM), borage oil, argan oil, onagra oil, linseed oil (0.5%), or vehicle (DMSO 0.1% for DHA or DMSO 0.5% for α-LA, borage oil, argan oil, onagra oil, and linseed oil). Final DMSO concentrations were the same for each compound and its control. At days 0, 1, 2, 3, and 4 (confluence) cells were fixed with formalin (10% in deionized water). Thereafter, cells were stained for 10 min with 0.3% Janus Green B dye (Sigma-Aldrich) at room temperature at constant stirring. Cells were then de-stained with water and dye was eluted with 0.5 M HCl at room temperature at constant stirring for 15 min. Absorbance values at 595 nm were determined using a microplate reader (Sinergy HT, Biotek, USA). Each compound was tested in three independent experiments. In other experiments, high power field images of human fibroblast cultures were taken at day 0 and 4 and the increase of area occupancy was determined by using the Image J software. We did not find gross differences between controls (increase in area occupancy: 58%) and FD patients (male *GLA* p.Y216fs*15 hemi, 57%; female *GLA* p.G183V heterozygous, 86%) and the male *NPC1* p.Q775P heterozygous, 93%).

Compounds

α-LA and docosahexaenoic acid (DHA) were purchased from Sigma-Aldrich (Deisenhofen, Germany) and were pre-diluted in DMSO giving a concentration of 1–500 μM (final concentration of DMSO 0.1–0.5%). TRAM-34, Senicapoc, SKA-31, and SKA-121 were a kind gift from Prof. Heike Wulff, Pharmacology at University of California, Davis. 13b ([3,5-bis[(3-fluoro-4-hydroxy-benzoyl)-oxymethyl]phenyl]methyl 3-fluoro-4-hydroxy-benzoate) was a kind from Prof. Robert Kiss, Laboratoire de Toxicologie, Institut de Pharmacie, ULB, Belgium. All other drugs were purchased from Sigma-Aldrich (Deisenhofen, Germany). Pure plant oils were purchased from a local herbalist's shop and pre-diluted in DMSO (final concentration of both, 0.5%).

RESULTS

Characterization of KCa3.1 in Murine 3T3-L1-Fibroblasts and Impact of the Omega-3 Fatty Acids, α-LA and DHA, on 3T3-L1 Mitogenesis *in vitro*

In murine 3T3-L1 fibroblasts, KCa3.1 currents developed after breaking into the whole-cell mode and concomitant infusion of Ca^{2+} via the patch-pipette. KCa3.1 currents displayed voltage-independence (for traces see **Figure 1A**, left panel; for additional details see Oliván-Viguera et al., 2015b). Pre-activation of the KCa3.1 currents and concomitant K+-efflux was accompanied by a shift of the reversal potential (E_{rev}) from basal (E_{rev}, $\approx -25 \pm 7$ mV, immediately measured after breaking into the whole-cell mode) to a more negative E_{rev} of $\approx -55 \pm 9$ mV.

The positive-gating modulators of KCa3.1, SKA-31 (1 μM, Sankaranarayanan et al., 2009) strongly potentiated the calcium-pre-activated currents by 19-fold and further shifted the E_{rev} to $\approx -76 \pm 3$ mV (for trace see **Figure 1A**, right panel, and summary data in **Figure 1B**, left panel). The classical KCa3.1 blocker, TRAM-34 (Wulff et al., 2000), virtually abolished the complete potentiated KCa3.1 currents (**Figure 1B**, right panel).

Regarding omega-3 fatty acids, α-LA and DHA, both at 10 μM, also abolished the complete SKA-31-potentiated KCa3.1 current and shifted E_{rev} back to more positive values, $E_{rev} \approx -11 \pm 4$ mV (for trace see **Figure 1A** and summary data **Figure 1B**, right panel).

QRT-PCR amplified mRNA-KCa3.1 in murine 3T3-L1 fibroblasts and expression levels were 0.02% of the reference gene, GAPDH, thus demonstrating intact gene expression of KCa3.1 in these cells.

Concerning the impact of α-LA on fibroblast proliferation, our Janus Green B colorimetric assay of cell proliferation revealed that α-LA concentration-dependently inhibited cell growth, with half-maximal inhibition at ≈100 μM (**Figures 2A,C**). However, concentrations ≥250 μM appeared to be cytotoxic in this assay. We also observed inhibition of cell growth when using DHA (**Figures 2B,C**), although DHA was seemingly more potent, with half-maximal inhibition at ≈10 μM.

Considering the reported beneficial effects of dietary supplementation of α-LA and α-LA-rich plant oils on autoimmune diseases and chronic inflammation such as multiple sclerosis, rheumatoid arthritis, psoriasis, chronic inflammatory bowel disease, or atopic dermatitis (Macfarlane et al., 2011; Wergeland et al., 2012; Yates et al., 2014) we were curious to learn whether α-LA-rich plant oils were also capable of inhibiting KCa3.1. The α-LA-rich linseed oil (from *Linum usitatissimum* and γ-LA-rich borage seed oil [*Borago officinalis* (Tso et al., 2012) produced significant channel inhibition at 1% while argan oil (from kernels of the argan tree (*Argania spinosa* L. (Charrouf and Guillaume, 2008) and onagra oil (from evening primrose seeds, *Oenothera biennis* L. (Fan and Chapkin, 1998)] with low levels of α-LA (<1%) and γ-LA (<10%), respectively, did not produce significant channel inhibition (**Figure 3A**).

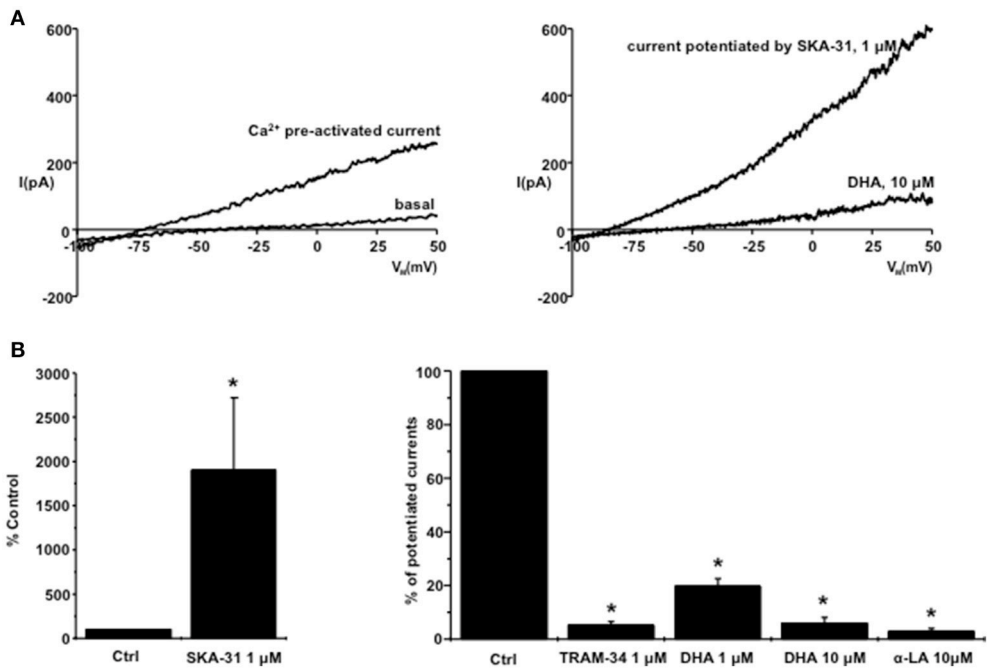

FIGURE 1 | Inhibition of KCa3.1 in murine 3T3-L1 fibroblasts by DHA and α-LA: (A) on left: Exemplary whole-cell current recordings of basal currents (after breaking into the whole-cell mode) and of pre-activated KCa3.1-currents by calcium. On right: SKA-31-potentiation of KCa3.1 currents (pre-activated by calcium) and inhibition of the complete potentiated current by DHA. **(B)** Summary data showing potentiation of calcium-pre-activated KCa3.1 currents by SKA-31 and inhibition of the complete SKA-31-potentiated KCa3.1 currents by DHA and α-LA. Data are means ± SEM. *$P < 0.05$, Student's *T*-test.

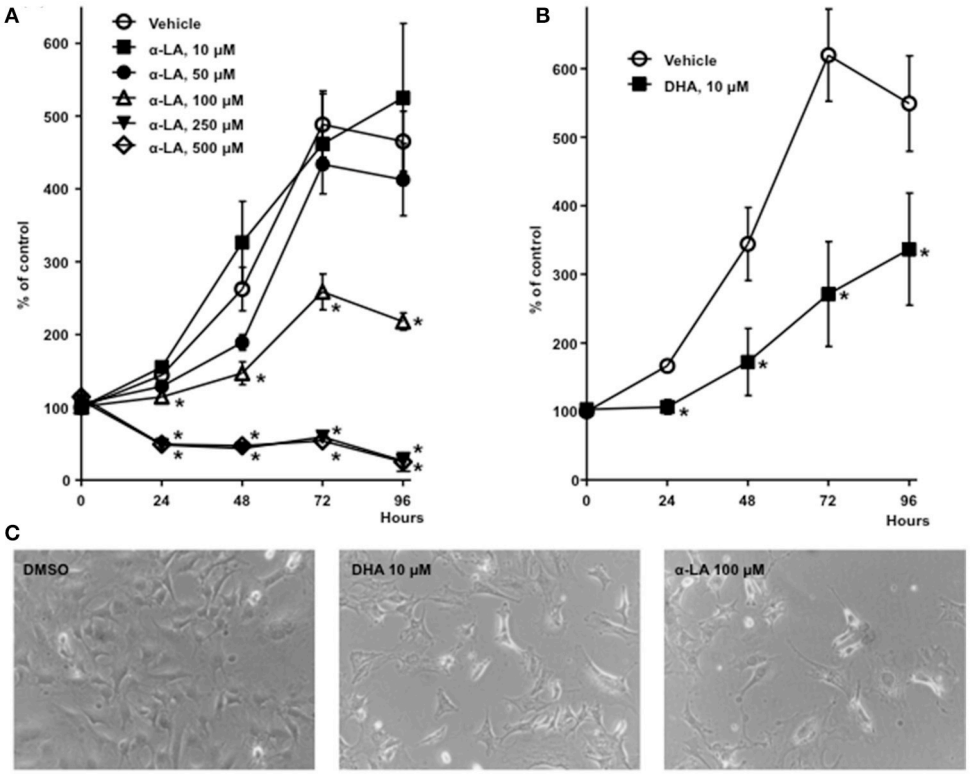

FIGURE 2 | Inhibition of 3T3-L1 fibroblast mitogenesis by α-LA and DHA: (A) Concentration-dependent inhibition of cell proliferation by α-LA. **(B)** Inhibition of proliferation by DHA. Data are means ± SEM. *$P < 0.05$, Student's *T*-tests. **(C)** High power field images of 3T3-L1 fibroblasts after 4 days of culture in the presence of DMSO (vehicle, 0.5%), DHA 10 μM and α-LA 100 μM.

Concerning 3T3-L1 fibroblast proliferation, linseed oil and borage oil produced significant inhibition of mitogenesis (**Figures 3B,C**) while onagra oil showed no significant effect on proliferation. Argan oil was found to be cytotoxic as cell numbers instantaneously decreased after application to cells (**Figures 3B,C**).

Characterization of KCa3.1 in Cultured Human Dermal Fibroblasts and Comparisons of Fibroblast KCa3.1 Functions in FD and NPC Patients

While KCa3.1 in murine fibroblast lines is well-characterized (Olivan-Viguera et al., 2013), information on KCa3.1 function and membrane expression in human dermal fibroblasts is scarce if compared to KCa3.1 in human fibroblasts in other tissues (Roach et al., 2014, 2015; Friebel et al., 2015). Moreover, altered KCa3.1 functions have been described in murine models of FD and FD patients (Choi et al., 2014, 2015; Choi and Park, 2016), suggesting a detrimental impact of lysosomal lipid accumulation on KCa3.1 protein expression or function.

Here, our mRNA-expression studies revealed substantial lower mRNA-KCa3.1 amounts in cell extracts from the male hemizygous FD-p.Y216fs*15 patient (**Figure 4**), the male NPC patient, but not from the female heterozygous FD-p.G183V patient.

Our patch-clamp studies detected KCa3.1 currents in human control fibroblasts with the same biophysical and pharmacology profile as in 3T3-L1 fibroblasts and of cloned human KCa3.1, i.e., calcium-activation, potentiation by SKA-31 (29-fold) or by SKA-121(13-fold), a related, more selective KCa3.1 activator (Coleman et al., 2014; for traces and summary data see **Figure 5A**). This potentiated current was virtually abolished by TRAM-34, Senicapoc, the negative-gating modulator, 13b (Olivan-Viguera et al., 2013), and α-LA (**Figure 5A**, right panel).

The comparison of SKA-potentiated and maximal KCa3.1-currents densities in the LSD patients revealed a clear trend toward lower functional KCa3.1 expression in the two hemizygous male FD patients and the male NPC patient but not in heterozygous female FD patients (**Figure 5B**): Mean KCa3.1 current densities were reduced to 5 and 20% of control in the two male FD, to 48 and 72% of control in the two female heterozygous FD patients, and to 13% in the male NPC patient.

Together, our data suggests that KCa3.1-gene expression and membrane expression of the channel are impaired in fibroblasts of hemizygous male FD patients and in the male NPC patients, while KCa3.1-functions in fibroblasts from heterozygous female FD patients are largely conserved.

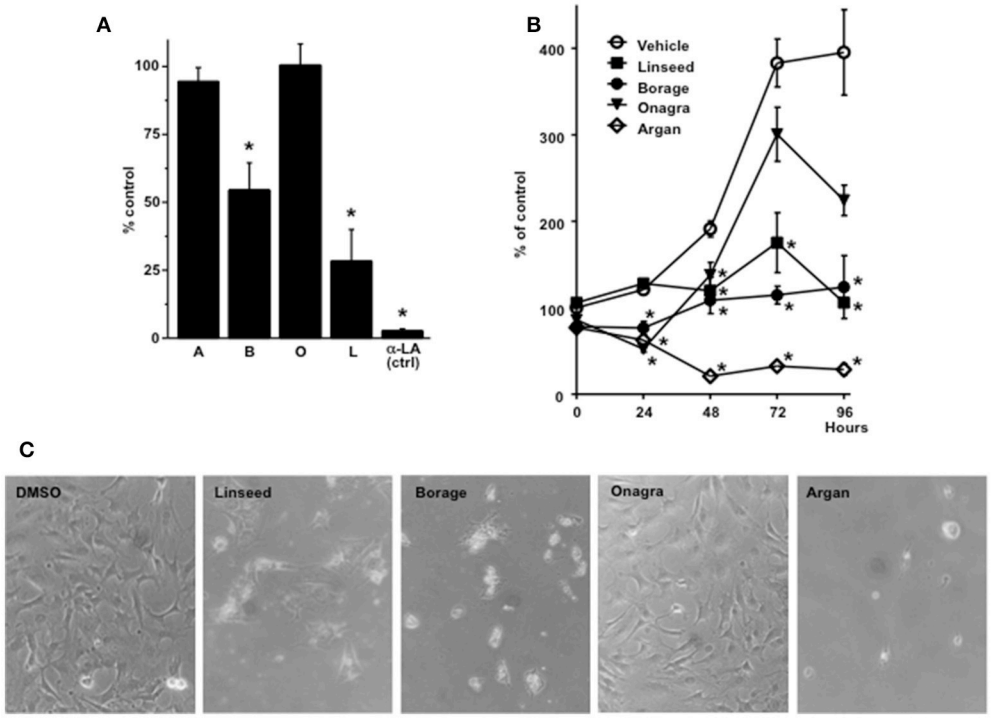

FIGURE 3 | Inhibition of KCa3.1 currents and 3T3-L1 fibroblast proliferation by plant oils. (A) Summary data showing the efficacy of different plant oils to inhibit KCa3.1 currents. A, argan oil; B, borage oil; O, onagra oil; L, linseed oil. α-LA at 10 μM served as positive control. The concentration of the oils was 0.5%. **(B)** Efficacy of plant oils to inhibit cell proliferation. Note that argan oil produced a decrease in cell number after 24 and 48 h, which was indicative of high cytotoxicity in this assay. Data are means ± SEM. $^*P < 0.05$, Student's *T*-test. **(C)** High power field images of 3T3-L1 fibroblasts after 4 days of culture in the presence of DMSO (vehicle), linseed oil, borage oil, onagra oil, and argan oil (0.5%).

DISCUSSION

KCa3.1 channel functions have been linked to abnormal cell proliferation, pathological tissue remodeling and fibrosis of a variety of organs, chronic inflammation, and autoimmune diseases (Wulff and Zhorov, 2008; Roach et al., 2013; Wulff and Köhler, 2013; Feske et al., 2015; Huang et al., 2015; Köhler et al., 2016). Accordingly, pharmacological inhibition of KCa3.1 has been suggested to be a treatment strategy for such disease states. In the present study we investigated: (1) the modulation of fibroblast KCa3.1 channels and fibroblast proliferation by omega-3 fatty acids, α-LA and DHA, as well as α-LA/γ-LA-rich and -low plant oils. (2) KCa3.1 functions in FD and NPC fibroblasts.

Here, we demonstrated KCa3.1 functions with the typical pharmacological fingerprint in murine 3T3-L1 fibroblasts and primary human dermal fibroblasts and showed inhibition of the channel by α-LA and DHA as well as potent anti-proliferative effects of these omega-3 fatty acids *in vitro*. Moreover, we demonstrated inhibition of KCa3.1 functions by α-LA- and γ-LA-rich plant oils and strong inhibition of 3T3-L1 fibroblast by linseed oil and borage oil, suggesting them as non-synthetic channel inhibitors with potential utilities for alleviating diseases states characterized by excessive cell proliferation, fibrosis, and chronic inflammation.

Impaired KCa3.1 functions have also been suggested to be a result of lipid overload in FD (Choi et al., 2014). Here, we demonstrated conserved but significantly reduced functional KCa3.1 channel membrane expression and mRNA expression in classical and atypical male FD patients and a NPC patient, KCa3.1 functions, suggesting impaired expression of KCa3.1 as potential biomarker of these LSDs.

Lipid regulation of K channels is a rather new field in ion channel research. So far, activation as well as inhibition of K channels has been described: Omega-6 and -3 fatty acids have been reported to activate KCa1.1 channels (a.k.a. BKCa encoded by the KCNMA1 and KCNMB1-3 genes; Kirber et al., 1992; Wei et al., 2005; Moreno et al., 2012). Likewise, the K2P channels, TWIK-2/2, TREK1/2, and TRAAK, were activated by omega-3/6 acids (Maingret et al., 1999; Wei et al., 2005; Nielsen et al., 2013). Regarding the KCa2/3 gene family, cloned KCa3.1 was, however, inhibited by omega-3/6 fatty acids (Hamilton et al., 2003; Kacik et al., 2014) and this inhibition required the inner-cavity lining amino acid residues T250 and V275 that are not present in corresponding sites in the above mentioned K channels families and in KCa2 channels. In the present study we provided new evidence for KCa3.1-inhibition by α-LA and also showed for the first time that also α-LA/γ-LA-containing plant oils (at 0.5%) were capable of efficiently inhibiting KCa3.1. However,

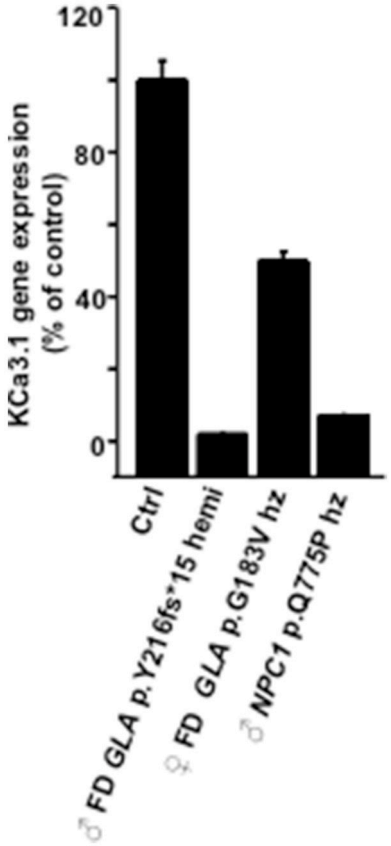

FIGURE 4 | Comparison of KCa3.1 gene expression and KCa3.1 current densities in fibroblasts from FD and NPC patients. Results from qRT-PCR showing gene expression of KCa3.1 m in FD and NPC patients relative to control (Ctrl). Data are means of triplicates ± SEM.

inhibitory activity clearly depended on high α-LA or γ-LA-content (>30%) since oils with low α-LA/γ-LA content failed to produce significant inhibition, which could be explained by the high contents of saturated or mono-saturated fatty acids that were not active on KCa3.1 but competed with α-LA as suggested previously for P450-derived metabolites of arachidonic acid such as 5,6-EET, 8,9-EET, 5,6-DiHETE, and saturated arachidic acid (Kacik et al., 2014).

The possible consequences of KCa3.1-inhibtion by α-LA for fibroblast proliferation were not established. Here, our study extended the current knowledge by showing that the α-LA- and γ-LA-rich plant oils, linseed oil and borage oil, respectively, exerted strong anti-proliferative activity. The γ-LA-low onagra oil was inefficient, a finding that further fostered the notion that for efficient targeting KCa3.1 and inhibition of cell proliferation a high-α-LA-/γ-LA was needed. Concerning argan oil, we cannot judge on possible anti-proliferative actions because the oil was found to be cytotoxic at the concentration used here.

Concerning the magnitude of inhibition of fibroblast proliferation the effect of α-LA and α-LA-rich oils were similar to reported effects of a synthetic negative-gating modulator of KCa3.1 and a classical pore blocker (Olivan-Viguera et al., 2013; Roach et al., 2015). However, here we do not wish to exclude other mechanisms by which α-LA and α-LA-/γ-LA-rich oils reduce fibroblast proliferation.

Dietary supplementation with α-LA and α-LA-containing oils has been suggested since long to have therapeutic utilities in several autoimmune diseases of skin and bowel, multiple sclerosis, and rheumatoid arthritis (for review see: Macfarlane et al., 2011; Wergeland et al., 2012). Some of these effects have been proposed to rely on interference with e.g., pro-inflammatory prostaglandin production/metabolism (for review see Yates et al., 2014).

Here, we suggest that inhibition of KCa3.1 could be an additional mechanism of action. KCa3.1 inhibitors have been shown to suppress pro-inflammatory cytokine production in immune cells and to reduce pathological organ remodeling and fibrosis in experimental models. Therefore, from the perspective of phytopharmacology, our data fostered the view that inhibition of KCa3.1 in psoriatic lesions of skin or the chronically inflamed bowel by topically or systemically applied α-LA/γ-LA-rich plant oils and/or dietary supplementation with α-LA/γ-LA-rich plant oils or by synthetic blockers (Wulff and Köhler, 2013; Christophersen and Wulff, 2015) could be an alternative and economic treatment for patients with mild forms of disease or an adjuvant treatment in more severely affected patients receiving immune suppressive therapy.

It should also be considered that the plant oils used in this study are of complex composition and the nature of the active compounds is still unclear. So, we do not wish to exclude that other compounds than α-LA caused some of the anti-proliferative effects on 3T3 fibroblasts. Yet, it was striking that the anti-proliferative effects correlated with the quantity of α-LA or γ-LA suggesting that α-LA/γ-LA played a major role here. In this regard, it is worth speculating that the cytotoxic effects caused by argan oil likewise rely on so far undefined compounds in this oil.

Dysfunction of lysosomal lipid metabolism/transport has recently been shown to impair KCa3.1-functions and mRNA-expression and reduced membrane surface expression by lowered phosphatidylinositol 3-phosphate as reason for these alterations (Choi et al., 2015; Choi and Park, 2016). In the present study, we provided additional evidence for such defective KCa3.1-regulation in FD fibroblasts and also in NPC fibroblasts from a male patient, which seemed predominantly to rely on impaired *de novo*-KCa3.1 gene expression, thus similar to that was found in earlier studies (Choi et al., 2015; Choi and Park, 2016). To the contrast, membrane expression—albeit at lower density—and activation was still intact in most cells, suggesting no major disturbances in membrane trafficking or function in the fibroblasts from FD patients investigated in our study.

Interestingly, the alterations were exclusively present in dermal fibroblasts of male FD patients with a classical missense mutation of this X-linked disease and an atypical complex intronic haplotype that have been reported to produce FD pathologies. In contrast, heterozygous female carriers with classical and atypical mutations, who may or may not develop clinically relevant symptoms depending on whether they have the mutated allele or the healthy allele as active copy, showed largely conserved functions of the channel in their dermal fibroblasts.

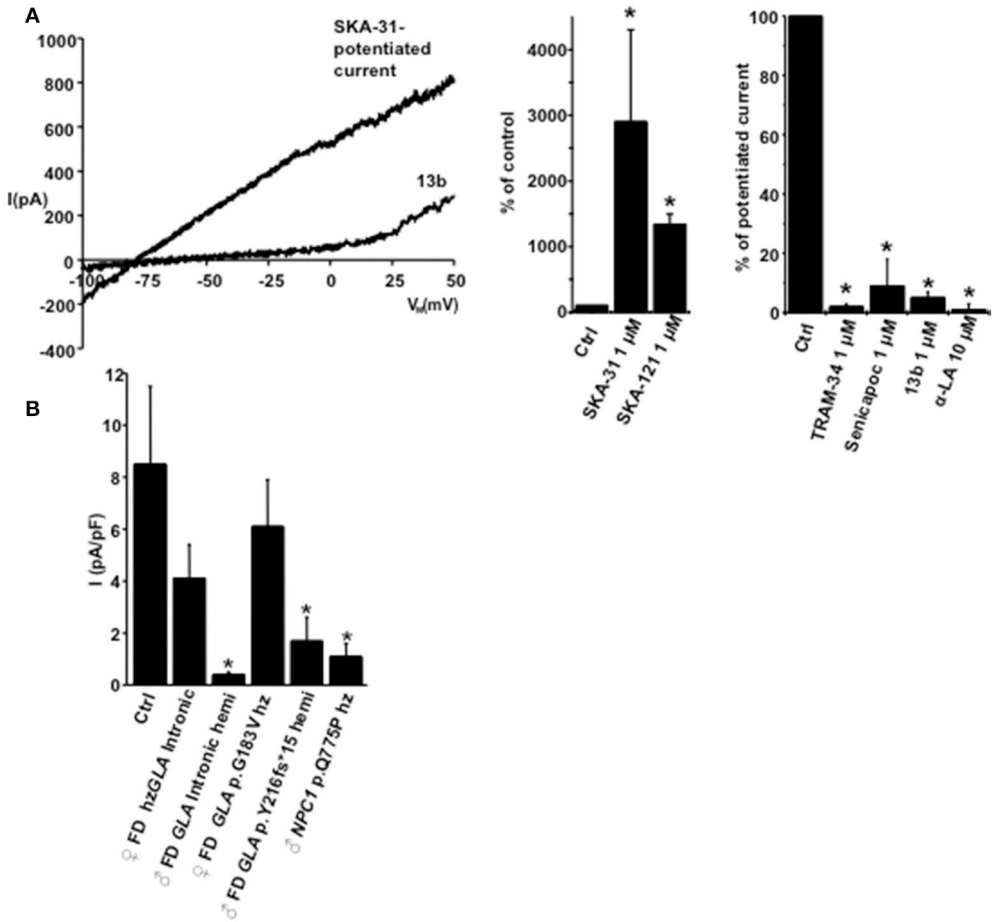

FIGURE 5 | Pharmacological characteristics of KCa3.1 in human fibroblasts. (A) On left: Exemplary traces showing the SKA-31 (1 μM)-potentiated fibroblast KCa3.1 current and inhibition of the complete SKA-31-potentiated KCa3.1-current by 13b (1 μM). In middle: Potentiation of K currents (background K currents or calcium-pre-activated by calcium) by SKA-31 ($n = 4$) and SKA-121 ($n = 5$). On right: Inhibition of the SKA-potentiated current by TRAM-34 ($n = 4$), Senicapoc ($n = 2$), 13b ($n = 10$), and α-LA ($n = 2$). **(B)** Mean SKA-potentiated KCa3.1-current densities in Ctrl, FD, and NPC patients. Percentage of cells displaying functional KCa3.1: Ctrl, 4 out of 4 cells (100%); ♀ FD *GLA* Intronic heterozygous, 13 out of 20 (65%); ♂ FD *GLA* Intronic hemizygous 1 out of 15 (7%); ♀ FD *GLA* p.G183V heterozygous, 5 out of 6 (83%); ♂ FD *GLA* p.Y216fs*15 hemizygous, 4 out of 6 (67%); ♂ *NPC1* p.Q775P heterozygous, 3 out of 5 (60%). Data in bar chart are means ± SEM, $^*P < 0.05$, Student's *T*-test.

Together, this suggested that low KCa3.1-mRNA expression in skin fibroblasts could be at least an additional biomarker of FD-disease or disease activity in male FD patients and in NPC patients.

Interestingly, in another study on KCa3.1 functions in type-1 and -3 Gaucher patients we found a defect in the physiologically occurring up-regulation of KCa3.1 during monocyte-to-macrophage differentiation (Oliván-Viguera et al., 2015a), further suggesting that KCa3.1 dysregulation is perhaps a more general feature of LSDs and cellular pathophysiology. But again, the pathophysiological mechanism and significance remain unclear.

At this point, we would like to remind the reader that KCa3.1 functions in disease states have been so far related to pro-proliferative and -inflammatory processes and pathological organ remodeling such as kidney and lung fibrosis (Wulff and Zhorov, 2008; Roach et al., 2013; Wulff and Köhler, 2013; Feske et al., 2015; Huang et al., 2015; Köhler et al., 2016). Accordingly, pharmacological inhibition of KCa3.1 has been proposed as pharmacological strategy to treat chronic inflammation and organ fibrosis. So, whether or not KCa3.1—albeit reduced—is drug target in FD and associated pathologies such progressive heart and kidney disease, aneurism, and angiokeratoma cannot be decided at present. Still, there is the possibility that inhibition of KCa3.1 by small molecules or α-LA-/γ-LA-rich oils as dietary supplement may delay disease progression in LSD patients who develop organ pathologies characterized by chronic inflammation and fibrosis.

There are some limitations concerning our patient study: First, the number of patients studied here was relatively small, which is a general limitation when studying these rare LSD with complex and varying clinical pathology. However, the observed differences in KCa3.1-expression and functions were still considerable (ca. 75% reduction) between controls and male FD patients. Here

additional, mRNA expression analysis in a larger number and other cohorts of patients will be needed to further establish KCa3.1 as biomarker of FD and/or other LSD. Whether these alterations were unique for FD disease remains unclear since a male NPC patient showed similarly reduced KCa3.1 functions. Secondly, in the present study we did not intent to reveal the pathomechanism linking defects in lipid metabolism and lipid overload to reduced KCa3.1 membrane functions, although lipid overload affecting *de novo*-gene expression is likely to be a major reason here.

In conclusion, the present study showed inhibition of KCa3.1 functions and fibroblast mitogenesis by the α-LA-/γ-LA-rich plant oils, *in vitro*. This suggests the utility of both as dietary supplement or topical ointments to target these pro-inflammatory and pro-mitogenic channels in cutaneous, neuronal or intestinal autoimmune disease.

AUTHOR CONTRIBUTIONS

AO and RK conceived the study, conducted research, analyzed data, and wrote the manuscript. JL, LL, and ÁLG conducted research and analyzed data. EA, YG, PG, AJG, JC, PI, conducted research on patients, analyzed data, provided materials, and compounds, and contributed to the writing of the manuscript.

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MINI REVIEW
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Regulation of Connexin-Based Channels by Fatty Acids

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In this mini-review, we briefly summarize the current knowledge about the effects of fatty acids (FAs) on connexin-based channels, as well as discuss the limited information about the impact FAs may have on pannexins (Panxs). FAs regulate diverse cellular functions, some of which are explained by changes in the activity of channels constituted by connexins (Cxs) or Panxs, which are known to play critical roles in maintaining the functional integrity of diverse organs and tissues. Cxs are transmembrane proteins that oligomerize into hexamers to form hemichannels (HCs), which in turn can assemble into dodecamers to form gap junction channels (GJCs). While GJCs communicate the cytoplasm of contacting cells, HCs serve as pathways for the exchange of ions and small molecules between the intra and extracellular milieu. Panxs, as well as Cx HCs, form channels at the plasma membrane that enable the interchange of molecules between the intra and extracellular spaces. Both Cx- and Panx-based channels are controlled by several post-translational modifications. However, the mechanism of action of FAs on these channels has not been described in detail. It has been shown however that FAs frequently decrease GJC-mediated cell-cell communication. The opposite effect also has been described for HC or Panx-dependent intercellular communication, where, the acute FA effect can be reversed upon washout. Additionally, changes in GJCs mediated by FAs have been associated with post-translational modifications (e.g., phosphorylation), and seem to be directly related to chemical properties of FAs (e.g., length of carbon chain and/or degree of saturation), but this possible link remains poorly understood.

Keywords: gap junction channel, hemichannel, connexon, pannexon, G-protein coupled receptor

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INTRODUCTION

Fatty Acids: General Characteristics

Fatty acids (FAs) are carboxylic acids classified into three groups based on the length of their aliphatic carbon tails (Layden et al., 2013). These include: (i) short (<6 carbons), (ii); medium (6–12 carbons); and (iii) long (>12 carbons) aliphatic chains (Talukdar et al., 2011; Layden et al., 2013). In addition, FAs are also classified by the number of double bonds present in their aliphatic chain: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs) (Poudyal and Brown, 2015). In turn, PUFAs can be classified into omega-3 (ω-3) and omega-6 (ω-6), based on the location of the last double bond (Schmitz and Ecker, 2008). Despite their structural similarities, ω-3 FAs generally cause biological responses opposing to ω-6 FAs (Senkal et al., 2007). Although traditionally the interest in FAs and their effect on human

health has been mainly related to cardiovascular diseases, it is now well accepted that FAs influence other diseases, including metabolic disorders such as type 2 diabetes and diseases with a significant inflammatory response (Calder, 2015).

In addition to serving as energy sources, FAs also impact the following: cell membrane properties, localization and activity of channels, receptors and transporters, and activation of intracellular signaling pathways through membrane receptors (Kim and Clapham, 1989; Schmitz and Ecker, 2008; Ichimura et al., 2009; Langelier et al., 2010; Calder, 2015). In this context, oleic acid (OA) and linoleic acid (LA) regulate the amount of GLUT4, decreasing both protein and mRNA levels in a concentration-dependent manner in skeletal muscle cells (Poletto et al., 2015).They also regulate their own metabolism, when for example palmitic acid (PA) and stearic acid (SA) stimulate intracellular lipid accumulation (Xiao et al., 2012). Additionally, FAs modulate connexin (Cx) and pannexin (Panx) functions, which play critical roles in cellular communication and the functional integrity of various organs and tissues (Sáez et al., 2005; Bedner et al., 2012). Therefore, FAs have profound effects on a myriad of cell functions, some of which could be related to the modulation of cell-cell communication mediated by Cxs and Panxs.

Connexins and Pannexins

Cxs are encoded by 20 genes in mice and 21 genes in humans (Söhl and Willecke, 2004; Bedner et al., 2012). In the endoplasmic reticulum (ER) and Golgi/Trans Golgi they assemble into hexamers, known as hemichannels (HCs) (Sáez et al., 2005; D'Hondt et al., 2009). Another possible configuration occurs when Cxs assemble into dodecamers formed by the serial docking of two HCs to form a gap junction channel (GJC), which connects the cytoplasm of contacting cells (Söhl and Willecke, 2004; Hervé and Derangeon, 2013). The role of GJCs in several cell functions depends on cell type, the Cx type expressed and the physiologic state of cells (e.g., quiescent or proliferating cells) (Jiang and Gu, 2005; Rackauskas et al., 2010; Bedner et al., 2012).

HCs form poorly selective channels (Chandrasekhar and Bera, 2012) that participate in paracrine and autocrine signaling, since they are pathways for releasing signaling molecules such as: ATP, PGE_2 and glutamate (Sáez et al., 2005). GJCs on the other hand enable the interchange of metabolites and second messengers between contacting cells. Hence, both GJCs and HCs are fundamental for several relevant functions under physiological and pathophysiological conditions (for further details see Sáez et al., 2003; Chandrasekhar and Bera, 2012; Retamal et al., 2015).

Panxs as Cxs present four transmembrane domains, two extracellular loops, one intracellular loop, and both N- and C-termini facing the cytosol. Both human and mouse genomes contain the following three Panx-encoding genes: Panx1, Panx2, and Panx3 (Baranova et al., 2004). Panx1 and Panx3 are composed of 6 subunits, whereas Panx2 is composed of 8 subunits (Ambrosi et al., 2010; Boassa et al., 2015). All Panxs form channels at the plasma membrane, but Panx3 also forms channels at the ER, where it seems to control calcium flux in this organelle (Ishikawa et al., 2011). As mentioned previously, Cxs and Panxs share a similar topology at the plasma membrane, and share certain functional properties. Thus, under normal conditions, both Cx HCs and Panx channels have very low open probabilities (Quist et al., 2000; Contreras et al., 2003), which can be increased (Chandrasekhar and Bera, 2012), for example, by raising the intracellular Ca^{2+} concentration (Locovei et al., 2006; De Vuyst et al., 2009). Another similarity between Cx HCs and Panx1 channels is that they are also permeable to ions and small signaling molecules (Panchin, 2005; Locovei et al., 2006).

REGULATION OF GAP JUNCTION CHANNELS BY FATTY ACIDS

Cxs are regulated by post-translational modifications, such as phosphorylation and S-nitrosylation (Retamal et al., 2006; Johnstone et al., 2012; D'Hondt et al., 2013). The regulation by FAs or their conjugated version however has received little attention. The first reports showed that OA (18:1), arachidonic acid (AA, 20:4) and docosahexaenoic acid (DHA, 22:6) are powerful inhibitors of GJCs in the heart, vascular smooth muscle cells, and liver epithelial cell lines (Hirschi et al., 1993; Hii et al., 1995).This inhibition effect was reversed upon washing the cells with a FA-free solution (Hirschi et al., 1993; Hii et al., 1995), suggesting that this response might be mediated either by direct FA interaction with the GJC or by activation of a membrane receptor. Additionally, the effect of OA was concentration-dependent, with a greater inhibitory effect at low OA concentrations (Hirschi et al., 1993). In cells derived from rat lacrimal glands, an inhibitory effect of PUFAs or SFAs over GJCs was also reported (Giaume et al., 1989). Thus, AA, LA (18:2) or lauric acid (12:0) induces GJC closure, an effect that is not prevented by inhibitors of AA metabolism (Giaume et al., 1989), excluding cyclooxygenase products as possible mediators (Schmilinsky-Fluri et al., 1997). Moreover, AA decreases junctional conductance in neonatal rat heart cells (Fluri et al., 1990). This effect is specific to the degree of AA saturation, because arachidic acid (a structural saturated analog of AA) showed no effect on GJC conductance (Fluri et al., 1990). AA-induced cell-cell uncoupling was also shown to be reversible and could be mediated by direct action on Cx proteins (Fluri et al., 1990). Alternatively, AA or other PUFAs, due to their amphipathic character, could interfere with GJC conductance by disturbing the lipid-protein interface (Schmilinsky-Fluri et al., 1997). The possibility that the FA-induced GJC-inhibition could be a consequence of changes in distribution or expression of Cxs has not been tested.

Another characteristic of FAs that could be significant in the regulation of GJCs is the length of their carbon chains. Research has proven that FAs of different lengths have unique chemical properties (Layden et al., 2013). In fact, perfluorinated FAs (PFFAs), which are FA analogs (Kudo et al., 2011), were shown to inhibit GJCs in a concentration-dependent manner in a liver epithelial cell line due to their aliphatic chains ranging from 7 to 10 carbons. PFFAs, on the other hand, with 2–5 or 16–18 carbon chain lengths had no effect on GJCs (Upham et al., 1998, 2009). Additionally, the inhibition by PFFAs was observed after 5 min of incubation, and involved an ERK-dependent pathway (Upham

et al., 2009). Moreover, the short-term inhibition induced by PFFAs in GJCs was lost after washing the cells, which suggests the involvement of an extracellular component. In this case, Upham et al. (2009) also reported on prevention with the presence of different protein kinase inhibitors. It is thus possible that PFFAs activate membrane receptors, which in turn activate signaling proteins such as protein kinases (Upham et al., 2009). It should also be considered that PFFAs with a carbon chain between 6 and 9 carbons increase the liver FA profile after 5 days of treatment, with specific increases in palmitoleic acid (PO, 16:1), OA (18:1) and eicosatrienoic acid (20:3) content (Kudo et al., 2011). Consequently, treatment under different exposure times may also induce different effects (e.g., acute vs. chronic treatments).

Inhibition of GJCs induced after 1 h treatment with LA was reversible in a rat liver epithelial cell line (Hayashi et al., 1997), and was probably mediated by an intracellular signaling pathway (such as PKA). In contrast, cell response to long-term treatments with LA (e.g., 6 days) was not recovered after washing out the extracellular solution. This may suggest that regulation could involve post-translational modifications at least for Cx43, as suggested by Hayashi et al. (1997). PUFAs also regulated GJC activity in a transfected cell line. For example, AA inhibited GJCs in Cx36 transfected HeLa cells, and GJC activity was restored after washout (Marandykina et al., 2013).

On the other hand, different FAs might act as activators, since they increase both GJC and HC activity. For example, in human endothelial cells, a reduction of Cx43 GJC coupling induced by hypoxia/reoxygenation was observed, but this effect was not detected in cells pretreated (for 2 days) with 10 μM EPA (20:5), a ω-3 PUFAs (Zhang et al., 1999, 2002). Accordingly, in rat astrocytes supplemented for 10 days with DHA, an increase in gap junctional communication was recorded (Champeil-Potokar et al., 2006). Also, the ω-6 gamma-linolenic acid (GLA, 18:3) increased Cx43 GJC activity in human vascular endothelial cells (Jiang et al., 1997).

Although it is not easy to characterize the effect of PUFAs on GJC activity, the effect of AA has been consistently associated to the same response: inhibition of GJC activity (Giaume et al., 1989; Fluri et al., 1990; Hii et al., 1995; Marandykina et al., 2013). But curiously, this type of response is absent in *Xenopus* oocytes, where AA does not affect GJCs formed by Cx46 (Retamal et al., 2011). This suggests that the signaling pathway associated to the AA response is missing in *Xenopus* oocytes. This situation could be a consequence of (1) different lipid compositions of the cell membrane (e.g., levels and/or distribution of cholesterol) or (2) absence of either specific extra- or intracellular signaling molecules (e.g., membrane receptors or protein kinases).

CONNEXIN MODIFICATIONS INDUCED BY FATTY ACIDS

PUFAs are known to induce changes in the expression, distribution, and post-translational modifications of Cx proteins, which have been found to be correlated with changes in GJC activity. In particular, GLA decreases Cx43 tyrosine phosphorylation in human vascular endothelial cells (Jiang et al., 1997), while OA enhances the phosphorylate state at Cx43-S368 in rat cardiomyocytes (Huang et al., 2004). This post-translational modification has been associated with the disassembly and/or closure of Cx43-GJCs (Huang et al., 2004; Solan and Lampe, 2014). Accordingly, DHA alone or with EPA increases Cx43 phosphorylation in rat astrocytes and vascular endothelial cells (Champeil-Potokar et al., 2006; Dlugosova et al., 2009; Radosinska et al., 2013). The participation of different protein kinases, such as PKA, PKC-epsilon, PI3K, AKT, Src, or MEK1/2, has been observed in this type of Cx regulation (Popp et al., 2002; De Vuyst et al., 2007; Figueroa et al., 2013; Radosinska et al., 2013). All these *in vitro* data corroborate what happens *in vivo*. Thus, under physiological conditions, the content of astrocytic Cx43 has been directly associated with DHA concentration in rat brain (Maximin et al., 2015).

Regulation of Cxs by other mechanisms has also been reported. In rat models of hypertensive and hypertriglyceremic diseases, treatment with DHA + EPA (between 3 weeks and 2 moths) restores the expression levels and distribution of Cx43 at the cell membrane (Fischer et al., 2008; Mitasíková et al., 2008; Dlugosova et al., 2009; Bacova et al., 2010). In rat neural stem cells, Cx43 increases only in lipid rafts (with no changes in total Cx43) after 3 days of treatment with DHA, suggesting that this effect was only due to a redistribution of Cx43 at the cell membrane (Langelier et al., 2010). This Cx43 response within lipid rafts is not so unexpected, because these membrane domains (which are cholesterol-rich) are associated with trafficking of membrane proteins (McIntosh et al., 2003; Sánchez et al., 2010), including some Cx types (e.g., Cx32, Cx36, Cx43, and Cx46), which are preferentially located inside the lipid rafts. Interestingly, other Cxs (e.g., Cx26 and Cx50) are preferentially found outside these membrane domains (Schubert et al., 2002; Defamie and Mesnil, 2012). Cholesterol levels seem to play an important role in the regulation of Cx43, as seen in a cell line derived from rat cardiomyocytes (H9c2 cells). This is because cholesterol enrichment reduced dye transfer through Cx43 GJCs, due to activation of a PKC-dependent signaling pathway that induces Cx43 phosphorylation at S368 (Palatinus et al., 2011; Zou et al., 2014). A second residue may also be involved, because phosphorylation on S365 inhibits phosphorylation of the amino acid residue S368 (Solan and Lampe, 2014). Moreover, the assembly of GJCs and their activity are regulated by the lipid composition of the cell membrane (Defamie and Mesnil, 2012). Interestingly, differences in lipid composition of the plasma membrane could explain the different cell- or Cx-dependent responses. For instance, in two different human hepatoma cell lines (HepG2 and SMMC-7721) the increase in GJC activity induced by retinoic acid is associated to an increase in the amount of Cx43 (HepG2 cells) or Cx32 (SMMC-7721 cells) (Yang et al., 2014).

HEMICHANNEL ACTIVITY AND FATTY ACIDS

The effects of FAs on Cx HC activity were only described in the last decade. Electrophysiological experiments have shown

that pro-inflammatory PUFAs induce a biphasic effect in Cx46 HCs expressed in *Xenopus oocytes*. Thus, LA increases Cx46 HC currents at a low concentration (0.1 μM), and decreases HC currents at a high concentration (100 μM). The maximum inhibitory effect was completed in 2 min, and the inhibition was reversible after washout (Retamal et al., 2011). This biphasic response was also suggested for Cx43, because 11,12-epoxyeicosatrienoic acid, which is a metabolic derivative of AA (Spector et al., 2004), transiently increased cell coupling followed by a sustained uncoupling in human endothelial cells (Popp et al., 2002).

LA was also shown to increase HC activity in HeLa cells transfected with Cx26, Cx32, Cx43, or Cx45 within a few minutes of esposure (Figueroa et al., 2013). LA also increased Cx43 HC activity in a cell line derived from human gastric epithelial cells (Puebla et al., 2016). In this case, the effect was mediated by the activation of GPR40 (a membrane receptor) and intracellular AKT-dependent signaling pathway (Puebla et al., 2016). The GPR40 receptor belongs to a group of G-protein-coupled receptors (GPCRs) that include 5 other membrane receptors, which are also activated by FAs (Talukdar et al., 2011). These receptors are proposed to play critical roles in various physiological and pathophysiological conditions (Miyauchi et al., 2010; Talukdar et al., 2011).

To date, the information on Panx regulation by FAs is limited. It has been shown that Panx1 and Panx3 are regulated by certain FAs. In human and rat liver cell lines, acute treatment with SFAs such as PA (16:0) and SA (18:0) increases Panx1 channel activity. This response contributes to ATP release, which finally induces apoptosis (Xiao et al., 2012). Apparently, the regulation of Panx1 channel activity by FAs would depend on the degree of unsaturation of the FA in question. For example, the monounsaturated versions of PA and SA [i.e., PO (16:1) and OA (18:1)] do not affect Panx1 channel activity. Conversely, a PUFA as AA (20:4) reduces the macroscopic membrane current of Panx1 channels expressed in *Xenopus* oocytes, and reduces the release of ATP (Xiao et al., 2012; Samuels et al., 2013). With regard to Panx3, treatment of L6 myotubes with palmitate, but not palmitolate, was observed to promote the release of a macrophage chemoattracting agent likely to be ATP-released through Panx3 channels, since it was abrogated after silencing Panx3 (Pillon et al., 2014).

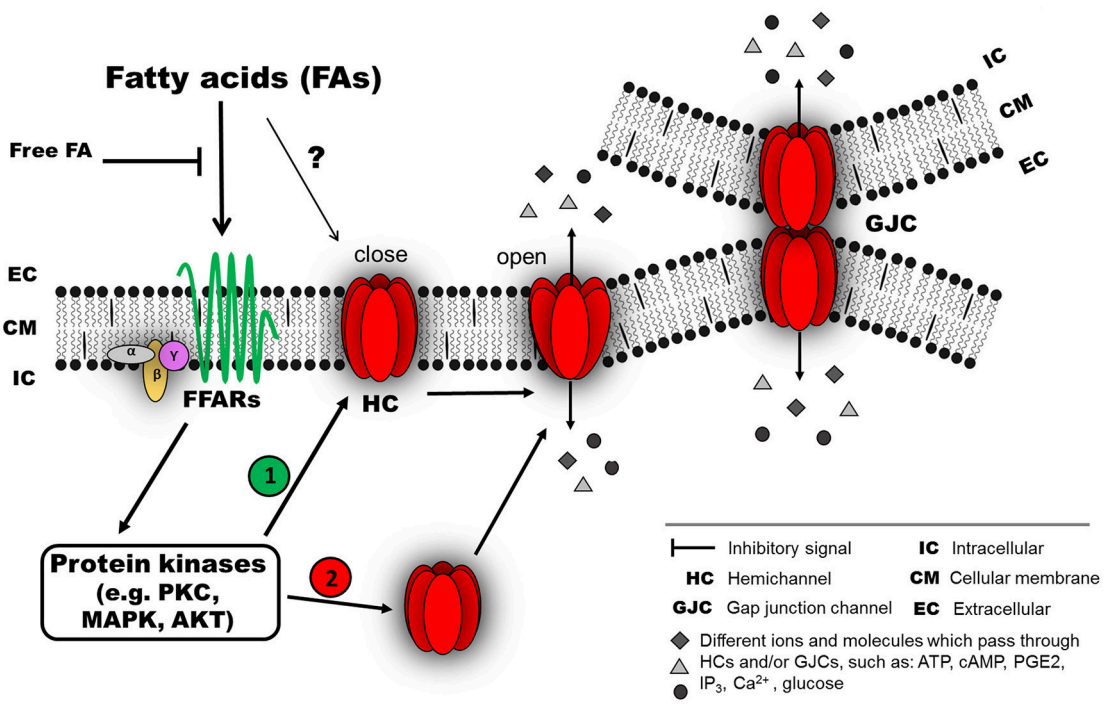

FIGURE 1 | Possible mechanism of connexin responses associated with acute exposure to fatty acids (FAs). FAs induce connexin (Cx) responses by interacting with membrane receptors, such as members of the G-protein coupled receptors (GPCRs), for example, GPR40 (free fatty acid receptor 1, FFAR1) or GPR120 (FFAR4), which are activated by medium- and long-chain FAs (Ichimura et al., 2014). The activation of these FFARs could involve an intracellular signaling pathway associated with different protein kinases (e.g., PKC, MAPK or AKT). Then, the activated kinase could induce at least two different effects: (1) modification of the open probability of Cx hemichannels (HCs) and/or gap junction channels (GJC), and/or (2) modification of Cx abundance in the cellular membrane (as HCs or GJCs) by changing the relative amount and distribution of intracellular Cx. In this model, the intracellular signaling associated with FFAR activation is lost after a wash out with a FA-free solution. A direct interaction between FAs and Cxs cannot be ruled out (?).

CONCLUSION

Since the effects of brief treatments with different FAs on GJC activity are reversible upon washout, it is likely that FAs act through membrane receptors and intracellular signaling pathways. There is evidence in support of the participation of membrane receptors in the short-term effect on Cx-based channels (both GJCs and HCs). In some cases, the FA-mediated effect requires the participation of protein kinases that could be activated downstream of different GPCRs (Osmond et al., 2012; Suire et al., 2012; Liang et al., 2015), such as FA receptors (Itoh et al., 2003; Hirasawa et al., 2005). In agreement with such evidence, several protein kinases have been identified to modify Cxs (Solan and Lampe, 2014; Pogoda et al., 2016). However, a direct interaction between FAs and Cxs cannot be ruled out (**Figure 1**). For long-term FA treatments, the effects are not reversible upon washout, and, therefore, a second mechanism may be involved, including regulation at the level of protein synthesis and/or protein redistribution in a cholesterol-dependent way (Gibbons, 2003). Other possible mechanisms that have scarcely been explored include regulation at the level of mRNA stability, mRNA synthesis (transcriptional regulation) or epigenetic regulation (Kiec-Wilk et al., 2011; Salat-Canela et al., 2015). Related to the latter, an increase in methylation of the Cx43 gene induced by AA in endothelial progenitor cells have been described (Kiec-Wilk et al., 2011) (**Figure 2**).

Cx regulation is critical for several cell functions and a large number of diseases can be attributed to changes in expression, function and/or properties of these proteins (Hills et al., 2015), it may be possible to suggest that the effect of FAs upon Cx-based channels can have an important impact in translational research. Thus, the uses of FAs that suppress HC activity in diseases where Cx HC activity is upregulated (e.g., ischemia reperfusion) could have important treatment benefits. Additionally, certain FAs could provide significant advantages in diabetic nephropathy, for instance, where they may restore the loss of GJC-mediated cell-cell communication within the nephron (Hills et al., 2015). Other examples where FAs may be used in addition to current therapies, and in which Cxs play important roles are lens cataracts (Beyer and Berthoud, 2014) and cancer, where Cxs have different (and controversial) types of participation (Aasen et al., 2016). Another advantage in the use of FAs for certain diseases is that many of them are harmless to humans. Alternatively, it may also be

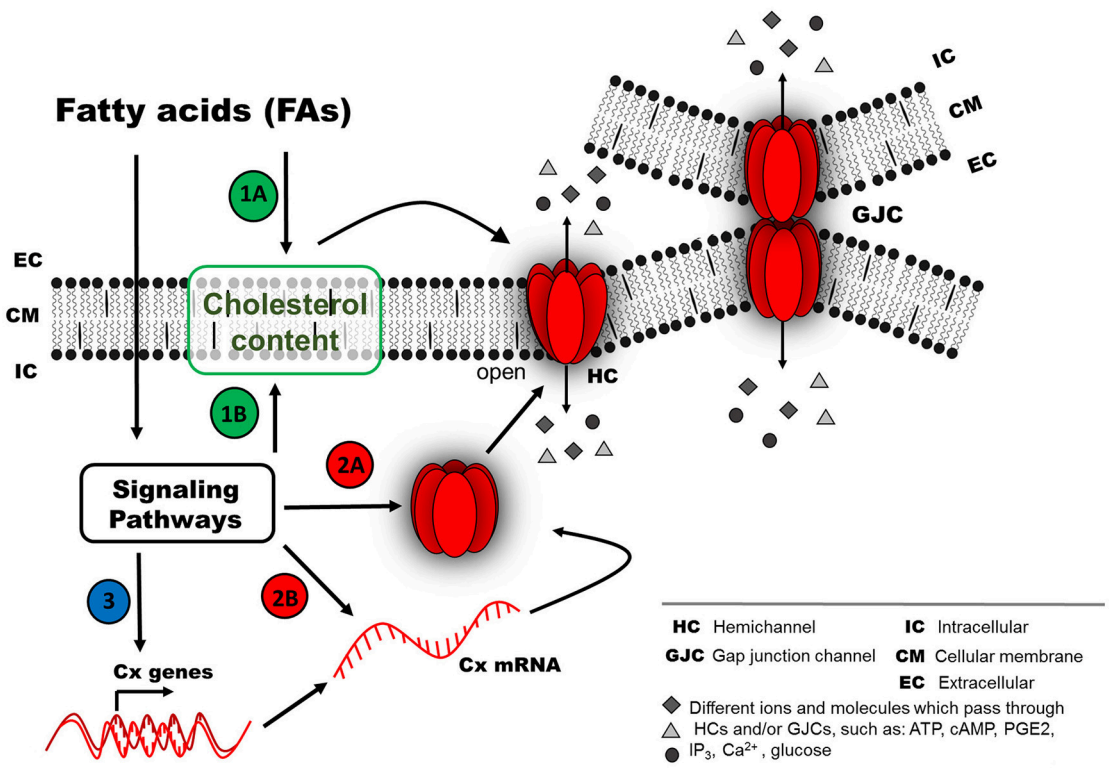

FIGURE 2 | Possible mechanism of connexin responses associated with chronic exposure to fatty acids (FAs). Chronic FA exposure could induce a long-term effect (second mechanism), because the FFARs could be inactivated due to long FA exposure (e.g., internalized or down-regulated). FA effects on the activity of Cx-based channels (as HCs or GJCs) probably involve different pathways, such as (1) regulation of Cx distribution at the cellular membrane by a cholesterol-dependent mechanism, either by direct action from the extracellular space (1A) or after activation of intracellular signaling pathways (1B). (2) Signaling pathways could modify Cx protein abundance due to changes in protein degradation rate (2A) or synthesis rate (2B). (3) Another option less studied is the regulation of mRNA stability or transcriptional activity of Cx genes (e.g., epigenetic regulation).

possible to develop modified FAs with higher specificities for Cx docking.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contributions to the work, and approved it for publication.

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Membrane Lipid Microenvironment Modulates Thermodynamic Properties of the Na^+-K^+-ATPase in Branchial and Intestinal Epithelia in Euryhaline Fish *In vivo*

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We have analyzed the effects of different native membrane lipid composition on the thermodynamic properties of the Na^+-K^+-ATPase in different epithelia from the gilthead seabream *Sparus aurata*. Thermodynamic parameters of activation for the Na^+-K^+-ATPase, as well as contents of lipid classes and fatty acids from polar lipids were determined for gill epithelia and enterocytes isolated from pyloric caeca, anterior intestine and posterior intestine. Arrhenius analyses of control animals revealed differences in thermal discontinuity values (*Td*) and activation energies determined at both sides of *Td* between intestinal and gill epithelia. Eyring plots disclosed important differences in enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) between enterocytes and branchial cells. Induction of n-3 LCPUFA deficiency dramatically altered membrane lipid composition in enterocytes, being the most dramatic changes the increase in 18:1n-9 (oleic acid) and the reduction of n-3 LCPUFA (mainly DHA, docosahexaenoic acid). Strikingly, branchial cells were much more resistant to diet-induced lipid alterations than enterocytes, indicating the existence of potent lipostatic mechanisms preserving membrane lipid matrix in gill epithelia. Paralleling lipid alterations, values of Ea_1, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the Na^+-K^+-ATPase were all increased, while *Td* values vanished, in LCPUFA deficient enterocytes. In turn, Differences in thermodynamic parameters were highly correlated with specific changes in fatty acids, but not with individual lipid classes including cholesterol *in vivo*. Thus, *Td* was positively related to 18:1n-9 and negatively to DHA. *Td*, Ea_1 and $\Delta H^{\ddagger}$ were exponentially related to DHA/18:1n-9 ratio. The exponential nature of these relationships highlights the strong impact of subtle changes in the contents of oleic acid and DHA in setting the thermodynamic properties of epithelial Na^+-K^+-ATPase *in vivo*. The effects are consistent with physical effects on the lipid membrane surrounding the enzyme as well as with direct interactions with the Na^+-K^+-ATPase.

Keywords: epithelial Na^+-K^+-ATPase, activation energy (*Ea*), entropy of activation ($\Delta H^{\ddagger}$), enthalpy of activation ($\Delta S^{\ddagger}$), n-3 long chain polyunsaturated fatty acids (LCPUFA), DHA (docosahexaenoic acid), oleic acid, membrane lipid microenvironment

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INTRODUCTION

In most epithelial tissues, the Na^+-K^+-ATPase is located at the basolateral membrane and provides the driving force for a variety of Na^+-dependent transport processes across the plasma membrane, which are critical for sustaining animal homeostasis (Schuurmans-Stekhoven and Bonting, 1981; Skou and Esmann, 1992). In marine teleost fish, the coordinated physiology of gill and intestinal epithelia is responsible for the mobilization of ingested seawater toward the submucosal vascular beds, and the extrusion of the excess of NaCl from ingested seawater, leading to a net balance of water intake (Evans, 1998; Evans et al., 2005). The key molecular components in the machinery for the elimination of NaCl against a dramatic electrochemical gradient reside in a specialized cell type, namely chloride cells, which are located in the branchial epithelia (Evans, 1998; Evans et al., 2005). Despite the complexity of iono- and osmoregulatory processes in marine fish, involved mechanisms are ultimately dependent on the exergonic activity of the Na^+-K^+-ATPase (Evans, 1998; Evans et al., 2005).

Previous studies performed in our laboratory have disclosed differences in the biochemical properties of the Na^+-K^+-ATPase activities along the intestinal tract of the gilthead seabream, *Sparus aurata* (Díaz et al., 1998; Almansa et al., 2001; Dópido et al., 2004). On the basis of the differential sensitivities to ouabain, calcium and to ionic strength, it was concluded that α-subunit of Na^+-K^+-ATPase expression varied along the intestinal tract with α_1 being ubiquitously expressed, but α_3-subunit only observed in distal regions (Almansa et al., 2001). Later on, we found that differences in thermodynamic properties existed between intestinal segments from gilthead seabream and that these differences were likely due to different phospholipid microenvironment rather than to differential expression of α-subunits (Almansa et al., 2003). The influence of the lipid microenvironment composition on the thermodynamic and kinetic properties of the Na^+-K^+-ATPase is pervasive and has been recognized for long (Wheeler and Whittam, 1970; Klingenberg, 1975; Brasitus, 1983; Cornelius and Skou, 1984; Yeagle et al., 1988; Muriana et al., 1992; Gerbi et al., 1993, 1994; Ventrella et al., 1993). From these early studies it soon became clear that the degree of unsaturation of membrane phospholipids, as well as membrane cholesterol contents, played modulatory roles on the kinetic features of the Na^+-K^+-ATPase, which were initially interpreted as secondary to differences in membrane fluidity. However, the precise molecular association between degree of phospholipid unsaturation, type of phospholipid, cholesterol-to-phospholipid relationships, on the membrane biophysics (and geometry) and dynamics of membrane-bound integral proteins is just starting to be unraveled. Studies using membrane models have demonstrated direct interactions between Na^+-K^+-ATPase α-subunits and specific membrane phospholipids and fatty acids, and that there exit interaction sites at the hydrophobic surfaces of both α-subunits and regulatory FXYD protein in the buried helixes exposed to the phospholipid bilayer (Arora et al., 1998; Cohen et al., 2005; Esmann and Marsh, 2006). At present, there exist solid evidence for direct and specific interactions of different phospholipids and cholesterol which affect both the stability and molecular activity of the Na^+-K^+-ATPase, with essential roles in physiological regulation linked to membrane lipid composition (Gerbi et al., 1993, 1994; Yeagle et al., 1988; Crockett and Hazel, 1997; Else and Wu, 1999; Cornelius, 2001; Almansa et al., 2003; Esmann and Marsh, 2006; Cornelius et al., 2015). Unlike model membranes, where lipid composition is set to specific compositions, usually containing few molecular lipid species, membranes from living cells display an enormous biochemical complexity with ∼10,000 different molecular species, likely depending on the organism and cell type, which, in turn, are subjected to continuous remodeling in response to intracellular and extracellular signals (Dowhan, 1997; Ernst et al., 2016). Important (and abundant) lipid molecules tightly associated to membrane physical properties in living cells are polyunsaturated fatty acids (LCPUFA). These fatty acids generally esterify glycerol backbone at *sn*-2 position in membrane phospholipids. Unlike saturates and monoenes, which can be synthesized by most vertebrates, including marine fish, LCPUFA cannot be produced (or are produced in very limited amounts) and their needs are mostly covered by their food intake. Amongst most important LCPUFA fatty acids, those belonging to the n-3 series (n-3 LCPUFA) and n-6 series (n-6 LCPUFA) are considered essential (Sargent et al., 1995; Díaz and Marín, 2013; Spector and Kim, 2015), and their deficiency are associated with a number of pathophysiological conditions and with the failure in adaptive responses (Bell et al., 1986; Gerbi et al., 1993, 1994, 1998; Sargent et al., 1995; Bogdanov et al., 2008; Russo, 2009; Díaz and Marín, 2013; Matsunari et al., 2013).

In the present study we have aimed to determine the influence of n-3 LCPUFA-deficient diets in the lipid composition of isolated intestinal and branchial epithelial cells of the gilthead seabream *in vivo,* and the extent to which such lipid modifications impact the thermodynamic properties of Na^+-K^+-ATPase from epithelial cells. The outcomes indicate that branchial epithelium is much more resistant to diet induced-LCPUFA depletion, in terms of phospholipid remodeling, than the intestinal counterparts. To the best of our knowledge, this is the first study demonstrating that the lipid composition of epithelial cells differs depending on their histological origin. Also, the results disclose severe changes in the thermodynamic behavior of the Na^+-K^+-ATPase that correlate with altered lipid environment-protein interactions *in vivo*.

MATERIALS AND METHODS

Animals, Diets, and Cell Preparation

Gilthead seabream (*S. aurata*) (average weight 400 g) were initially reared at the National Institute of Oceanography of Tenerife (Spain) in seawater ($35^\circ/_{oo}$) at 20°C and fed commercial fish pellets (CONTROL), containing 2.07% n-3 LCPUFA (dry weight basis, DWB). A subset of specimens were then reared in the Atlantic Ocean (Los Gigantes, Southwest coast of Tenerife, Spain) in parallel tanks containing 10 individuals each, and fed once a day with an amount of pellets equivalent to 1% their biomass of the control diet (CONTROL) or a LCPUFA-deficient diet (DEFICIENT) for 6 months. The experimental

diet containing no n-3 LCPUFA and very low amounts of n-3 PUFA was based on olive oil as lipid source, and obtained from Stirling University's Fish Nutrition facility (UK). Detailed composition of diets is shown in **Table 1**. After decapitation, intestinal segments were isolated from pyloric caeca and from the proximal and distal portions of the intestine as described previously (Almansa et al., 2001). For branchial epithelia, animals were first perfused through continuous ventricular injection of Ringer physiological solution to remove blood cells from gills. All tissues were rinsed in ice-cold Ringer solution, and submitted to epithelial cells isolation following the procedures described in detail in Dópido et al. (2004). Isolated cells were immediately placed in the homogenization solution containing 50 mM sucrose, 20 mM TRIS, 1 mM EDTA, 1 mM of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), pH 7.5 (adjusted with TRIS/HCl) and kept at 4°C. Once homogenized, samples were stored in 1 ml aliquots at −80°C until analysis. All experimental manipulations were carried out following the procedures approved by the ethics committee (Comité de Ética de la Investigación y de Bienestar Animal: CEIBA) from Universidad de La Laguna.

TABLE 1 | Composition of control and PUFA-deficient diets.

	Control	Deficient
Protein	40	38
Lipids	21	19
Celulose	3	5
Humidity	9	10
Ash	7	9

Data in % Weight

Fatty acids

	Control	Deficient
14:0	6.8	tr
16:0	18.9	11.8
16:1	7.6	1.4
18:0	3.7	2.9
18:1	12.7	72.1
18:2 n-6	4.3	9.6
18:3 n-3	1.4	0.6
18:4 n-3	2.9	–
20:1	2.4	0.4
20:2 n-6	0.2	tr
20:4 n-6	0.7	tr
20:4 n-3	0.7	tr
20:5 n-3	12.6	tr
22:1	2.2	0.4
22:5 n-6	0.4	tr
22:5 n-3	1.4	tr
22:6 n-3	13.2	tr
Monoenes	26.3	74.3
Saturates	30.7	14.7
n-3 PUFA	32.2	0.9
n-6 PUFA	5.9	9.8
n-3 LCPUFA	27.9	tr
n-6 LCPUFA	1.1	tr
n-3/n-6	5.5	0.1

Data in % area. PUFA: polyunsaturated fatty acids (≥2 double bonds). LCPUFA: Long-chain polyunsaturated fatty acids (≥18 carbons and ≥2 double bonds).

ATPase Assays

Na^+-K^+-ATPase activities were measured in triplicate as the difference in inorganic phosphate (Pi) production from ATP in the presence or absence of 1 mM ouabain, under steady-state conditions, as described previously (Ventrella et al., 1990, 1993; Díaz et al., 1998; Almansa et al., 2001). Briefly, 25 μl of protein suspension, containing 50–100 μg protein, were added to test tubes containing 1 ml of incubation media (25 mM HEPES, 200 mM NaCl, 10 mM KCl, 5 mM MgCl2, and adjusted to pH 7.4 with TRIS) and allowed to preincubate for 5 min in a temperature-controlled water-jacketed chamber set at the desired temperature. The reaction was started by the addition of 50 μl (5 mM final concentration) of vanadate-free ATP and incubated for 10 min at different temperatures ranging from 4 to 50°C under continuous agitation. The amount of Pi produced was determined by the method of Forbush (1983). Accordingly, at the end of the incubation period, the reaction was stopped by adding 1 ml of an ice-cold solution containing 2.8% ascorbic acid, 0.48%, ammonium heptamolybdate, 2.8% SDS and 0.48 M HCl, and the tubes were placed at 4°C for 10 min. Afterwards, the reaction was developed by incorporating 1.5 ml of 2% sodium citrate, 2% sodium-m-arsenite and 2% acetic, incubated at 37°C for another 10 min, and the absorbance read at 705 nm. Na_2HPO_4 was used as Pi standard for calibration curves. Corrections for unspecific ATP hydrolysis were made by measuring the amount of Pi liberated in the absence of protein samples at each temperature tested. Specific Na^+-K^+-ATPase activities were expressed as μmol Pi/mg prot.hr.

Lipid Analysis

Gill and intestinal epithelial homogenates were also submitted to lipid analyses. For these purposes, cell collections were homogenized in chloroform/methanol (2:1 v/v) containing 0.01% of butylated hydroxytoluene as antioxidant, and then the organic solvent was evaporated under a stream of nitrogen. Lipid contents were determined gravimetrically. Lipid extracts were re-dissolved in chloroform/methanol (2:1 v/v) and stored in 1 mL glass vials in a nitrogen atmosphere free of O_2 at −20°C until analyses.

Lipid classes were separated by one-dimensional double development high performance thin layer chromatography (HPTLC) as described elsewhere (Olsen and Henderson, 1989; Martín et al., 2006) and quantified by scanning densitometry using a Shimadzu CS-9001PC dual wavelength flying spot scanner. The polar lipid fraction (PL) was separated from neutral lipids by silica sep-pak cartridges and then subjected to acid-catalyzed transmethylation to yield fatty acid methyl esters (FAME) (Christie, 1982).

FAME were separated and quantified using a Shimadzu GC-14A gas chromatograph equipped with a flame ionization

detector, an integrator and a fused silica capillary column Supelcowax TM 10 (30 m × 0.32 mm I.D.). Individual FAME were identified by reference to a multi-standard mixture which included authentic standards from marine fish (Supelco, Bellefonte, USA) and further confirmation of identity carried out by mass spectrometry when necessary.

Statistics and Calculations

All results are expressed as means ± SEM for, at least, four different determinations. Experimental data were submitted to one-way ANOVA followed by Tukey's test or to Kruskall-Wallis analysis followed by Mann-Withney *U* test, were appropriate. Correlation and determination coefficients were obtained by Pearson's approach. Multivariate analyses were performed using Principal Component Analyses (PCA). Statistical calculations were performed using SPSS (v.15.0 SPSS Inc., Chicago). Estimations of regression equations (lineal and exponential) and related parameters were performed by non-linear regression analysis tools using *Sigma Plot* software (Jandel Scientific, San Rafael, CA). A *p*-value below 0.05 was considered to achieve statistical significance.

Materials

Ouabain, sodium dithionite, vanadate-free Na_2ATP, HEPES, EDTA, TRIS, type IV Collagenase and PMSF were purchased from Sigma-Aldrich (Biosigma, Spain). Dimethylsulfoxide and HPTLC plates were obtained from Merck (Germany). Silica sep-pak cartridges were supplied by Millipore (Milford, MA). All reagents were analytical grade.

RESULTS

Lipid Profiles in Isolated Gill and Intestinal Epithelial Cells from Control Fish

Lipid composition of gill epithelia and isolated enterocytes obtained from animals fed control diets are shown in **Table 2**. It can be observed that neutral and polar total lipid contents as well as individual lipid classes are rather homogeneous between intestinal segments. Only phospahtidylglicerol (PG, lowest in posterior intestine) and free fatty acids (FFA, lowest in anterior intestine) significantly differ between regions. However, it turns out that lipid profiles in epithelial cells from gill origin notably differ from those of enterocytes. Thus, gill epithelial cells contain lower amounts of phosphatidylcholine (PC), phosphatidylinositol (PI), phospahtidylglycerol (PG), phosphatidylserine (PS), phosphatidylethanolamine (PE), and as a consequence, total polar lipids (TPL), as well as higher levels of cholesterol (CHO), sterol esters (SE), free fatty acids (FFA) and resultant total neutral lipids (TNL) compared to enterocytes (**Table 2**).

Regarding fatty acids from polar lipids, significant differences between enterocytes were observed for stearic acid (18:0), linoleic acid (18:2n-6) and docosapentaenoic acid (DPA, 22:5n-6), which were higher in posterior intestine. However, when compared with gill epithelium significant differences in most fatty acids were observed. Thus, levels of palmitoleic acid (16:1), linoleic acid (18:2n6), linolenic acid (18:3n-3), and total monoenoic and total n-6 fatty acids were higher than in enterocytes, while contents of eicosatetraenoic acid (20:4n-3), eicosapentaenoic acid (20:5n-3), docosahexaenoic acid (22:6n-3) and total n-3 LCPUFA were notably lower than in the enterocytes within the same animals. Consequently, the 18:1/n-3 LCPUFA relationship, was significantly higher in gill epithelial cells.

The results presented so far reveal that lipid profiles in gill epithelia substantially differ from that in enterocytes. This was further confirmed by multivariate analyses using the principal component analyses (PCA). The results of the factor scores plot shown in **Figure 1** indicate that the three populations of enterocytes were clustered together while the gill population was clearly segregated (**Figure 1A**). The variables explaining most variance (overall variance 76.90%) were 18:1n-9, 20:4n-6 (ARA, arachidonic acid), linolenic acid (18:3n-3), myristic acid (14:0), which were positively related to PC1, and DHA, stearic acid (18:0) and 20:1 which were negatively related to PC1 (**Figure 1A**). PC2 was positively correlated to three n-6 fatty acids (18:2n-6 or linoleic acid, 20:3n-6, and 22:5n-6) and negatively to palmitic acid (16:0). Indeed, differences in PC2, allowed a certain differentiation between enterocytes, with caecal and posterior enterocytes appearing as opposite clouds, and anterior enterocytes positioned between them (**Figure 1B**).

Effects of n-3 PUFA-Deficient Diets on Lipid Profiles of Isolated Gill and Intestinal Epithelial Cells

In the next experiment we aimed to determine the impact of dietary n-3 PUFA deficiency in the lipid composition of the four populations of epithelial cells. The results in **Table 3** indicate that TPL were reduced by the treatment in enterocytes (at least in caecal and anterior enterocytes). This effect was due to general reductions of PC, PS, PI, and PE in deficient animals although differences only scarcely reached statistical significance. However, none of these trends were observed in gill epithelia. Regarding total neutral lipids, a generalized increase in the contents of SE, triacylglycerides (TAG) and TNL was observed in enterocytes from deficient animals irrespective of the intestinal origin. As in the case of TPL, none of these effects were observed in branchial cells.

Profiles of fatty acids in polar lipids shown in **Table 3**, also indicate severe changes in enterocytes from deficient animals. Thus, a dramatic reduction of DHA occurs in all enterocyte preparations, reflecting the composition of deficient diets. Furthermore, the depletion of DHA in enterocytes was accompanied by pervasive increase in oleic acid (18:1n-9), linoleic acid and total monoenoic fatty acids. Strikingly, none of these changes appear to affect gill epithelium, as only few minor fatty acids were significantly changed.

Given that main differences between enterocytes from LCPUFA deficient animals were observed for fatty acids, we next performed multivariate analyses using PCA. The results illustrated on **Figure 1C** revealed that two principal components explained 64.1% of overall variance, with PC1 being positively related to saturates (mainly 14:0, 16:0, and 18:0) and negatively

TABLE 2 | Lipid classes and total polar lipid fatty acid composition of isolated epithelia from gilthead seabream reared under standard conditions.

	Pyloric caeca	Anterior intestine	Posterior intestine	Gill epithelia
LIPID CLASSES				
LPC	0.27 ± 0.46	0.18 ± 0.21	0.27 ± 0.65	0.20 ± 0.29
SM	1.85 ± 0.56	2.08 ± 0.70	2.10 ± 0.86	1.04 ± 0.44
PC	19.38 ± 2.46a	17.49 ± 2.13a	17.77 ± 1.87a	4.21 ± 1.50b
PS	3.36 ± 0.80a	3.23 ± 0.56a	2.81 ± 0.89ab	1.08 ± 0.46b
PI	4.66 ± 0.57a	4.30 ± 0.77a	3.72 ± 0.70a	1.03 ± 0.21b
PG	5.31 ± 0.58a	4.31 ± 0.95a	3.11 ± 0.78b	1.02 ± 0.77c
PE	12.13 ± 2.14a	10.97 ± 1.86a	10.27 ± 2.54a	3.03 ± 2.10b
TPL	46.96 ± 6.48a	42.56 ± 5.46a	42.02 ± 6.56a	11.61 ± 4.91b
MAG	1.93 ± 1.14b	1.92 ± 1.12b	1.41 ± 1.24b	7.39 ± 3.82a
DAG	0.00 ± 0.00	0.00 ± 0.00	0.27 ± 0.67	0.52 ± 0.74
CHO	18.95 ± 2.24b	18.42 ± 1.64b	19.46 ± 0.57b	25.83 ± 2.11a
FFA	1.78 ± 1.83b	5.33 ± 3.64a	7.39 ± 4.53a	14.44 ± 6.08a
TAG	23.24 ± 5.16b	24.48 ± 2.50b	23.16 ± 4.14b	33.46 ± 1.24a
SE	5.57 ± 1.66	5.61 ± 2.81	6.06 ± 2.00	3.22 ± 0.36
TNL	53.04 ± 6.48b	57.44 ± 5.46b	57.98 ± 6.56b	84.86 ± 6.72a
LT (μg/mgprot)	554 ± 60a	375 ± 49b	345 ± 53b	560 ± 80a
FATTY ACIDS				
14:0	1.07 ± 0.53b	0.44 ± 0.24b	0.32 ± 0.21b	2.05 ± 0.11a
16:0	21.99 ± 1.38	21.34 ± 1.09	21.05 ± 0.92	22.08 ± 0.75
16:1	0.77 ± 0.34b	0.45 ± 0.17b	0.57 ± 0.22b	3.73 ± 0.64a
18:0	10.31 ± 0.79b	11.04 ± 0.84ab	12.11 ± 0.23a	8.56 ± 0.45b
18:1 n-9	9.84 ± 0.62b	10.70 ± 0.27b	10.10 ± 0.87b	14.85 ± 0.21a
18:2 n-6	2.49 ± 0.05	2.49 ± 0.03	3.21 ± 0.40	2.85 ± 0.01
18:3 n-3	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.17 ± 0.00a
18:4 n-3	0.09 ± 0.08	0.12 ± 0.10	0.13 ± 0.11	0.17 ± 0.02
20:1	1.41 ± 0.14b	1.98 ± 0.40b	1.49 ± 0.13b	0.45 ± 0.16a
20:2 n-6	0.36 ± 0.07	0.30 ± 0.03	0.20 ± 0.23	0.00 ± 0.00
20:4 n-6	2.59 ± 0.27b	2.41 ± 0.18b	2.62 ± 0.25b	5.10 ± 0.43a
20:4 n-3	0.48 ± 0.04a	0.53 ± 0.06a	0.54 ± 0.03a	0.39 ± 0.05b
20:5 n-3	6.15 ± 1.55b	6.32 ± 1.94b	6.14 ± 1.13b	9.15 ± 0.38a
22:1	0.25 ± 0.05a	0.40 ± 0.27a	0.30 ± 0.16a	0.00 ± 0.00b
22:5 n-6	0.46 ± 0.04	0.56 ± 0.07	0.60 ± 0.05	0.57 ± 0.10
22:5 n-3	2.25 ± 0.36a	2.47 ± 0.23a	2.55 ± 0.21	2.53 ± 0.32
22:6 n-3	34.13 ± 2.17a	35.04 ± 0.79a	32.70 ± 1.50a	21.69 ± 0.33b
TOTALS AND INDEXES				
Monoenes	12.29 ± 0.90b	13.53 ± 0.53b	12.47 ± 0.58b	20.19 ± 0.21a
Saturates	33.69 ± 1.71	33.35 ± 1.72	34.03 ± 1.14	34.06 ± 0.67
n-6	6.03 ± 0.38b	5.98 ± 0.09b	6.86 ± 0.53b	8.85 ± 0.57a
n-3 LCPUFA	43.03 ± 1.93a	44.38 ± 1.67a	41.95 ± 1.33a	33.76 ± 1.08b
18:1/n-3 LCPUFA	0.16 ± 0.00b	0.17 ± 0.01b	0.17 ± 0.01b	0.44 ± 0.01a
UI	248.96	256.74	244.40	194.35

Different letters in the same row state for statistical differences with p-values below 0.05. LPC, Lyso-phosphatidylcholine; SM, Sphingolipids; PC, Phosphatidylcholine; PS, Phosphatidylserine; PI, Phosphatidylinositol; PG, Phosphatidylglycerol; PE, Phosphatidylethanolamine; TPL, Total Polar Lipids; MAG, Monoacylcerides; DAG, Diacylcerides; CHO, Cholesterol; TAG, Triacylcerides; FFA, Free fatty acids; SE, Sterol esters; TNL, Total Neutral Lipids; TL, Total Lipids; UI, Unsaturation Index. With the exception of 18:1/n-3 LCPUFA ratio, UI and TL, all data are expressed as % of total lipid. TL is expressed as μg lipids/mg total protein.

to polyunsaturated fatty acids (mainly 20:4n-6, 20:5n-3, and 22:6n-3). Interestingly, PC2 was positively related to 18:1n-9, but PC1 was insensitive to this fatty acid, despite large differences between groups. Plotted factor scores revealed that deficiency-induced changes in fatty acids composition of phospholipids was so dramatic that two clusters corresponding to control and deficient enterocytes were plainly segregated (**Figure 1D**). Further, when factor scores were plotted for each intestinal segment, it was observed that irrespective of the intestinal origin, the three groups of enterocytes

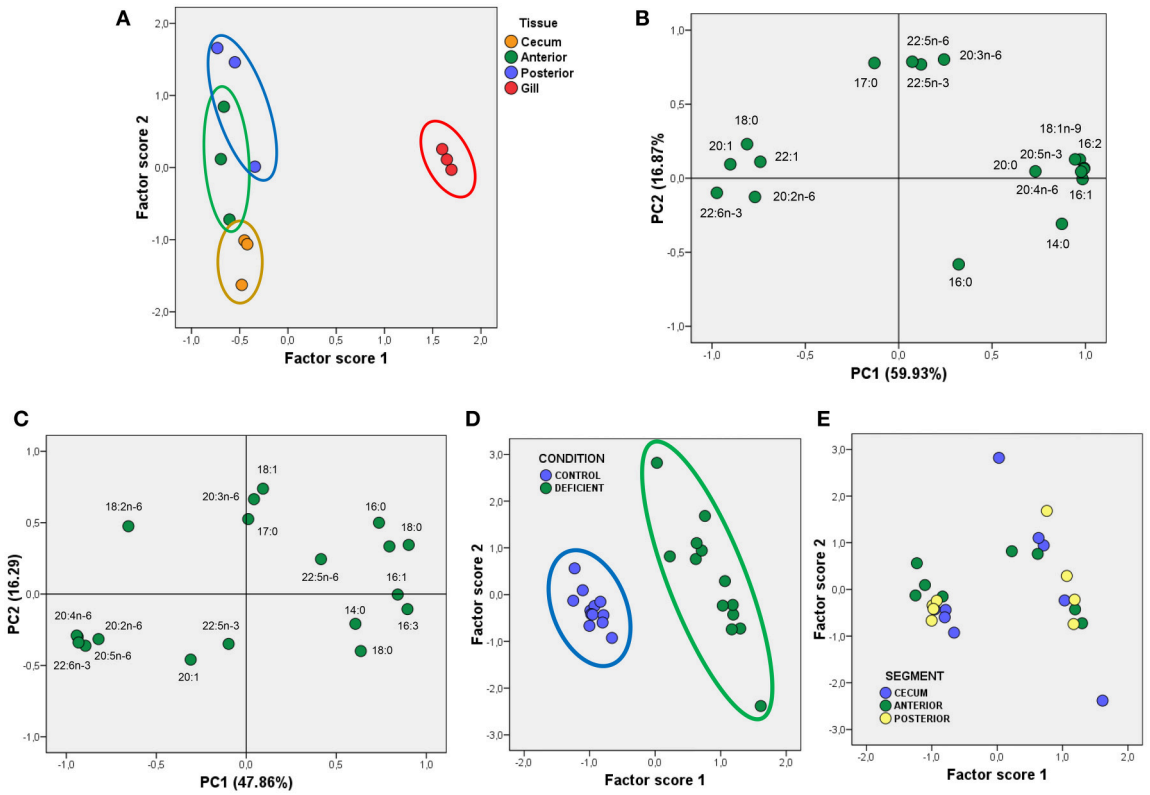

FIGURE 1 | Multivariate analyses using PCA of fatty acids from total polar lipids in gilthead seabream epithelia. (A) Scatterplot for Factor scores 1 and 2, and grouped to identify different epithelial sources in control animals. **(B)** Factor loadings for Principal Components 1 and 2 (PC1 and PC2) obtained for fatty acids from TPL in control animals. The fraction of total variance explained by each principal component is indicated in parentheses. **(C)** Factor loadings for Principal Components 1 and 2 (PC1 and PC2) obtained for fatty acids from TPL in enterocytes from Control and LCPUFA-deficient animals. As in **(B)**, the fraction of total variance explained by each principal component is indicated in parentheses. **(D,E)** Scatterplots for Factor scores 1 and 2 grouped to identify different diets **(D)**, and enterocyte sources **(E)**. For details see corresponding results section.

are represented in both CONTROL and DEFICIENT clouds (**Figures 1D,E**).

Thermodynamic Properties of Na^+-K^+-ATPase in Isolated Enterocytes and Branchial Cells

We next examined the temperature-dependence curves for the Na^+-K^+-ATPase reaction rates from the three groups of enterocytes and branchial cells under control conditions. Arrhenius plots for the Na^+-K^+-ATPase from intestinal and branchial preparations along with the Eyring plots (in the insets) are shown in **Figure 2**. Arrhenius plots indicate that the overall reactions of ATP hydrolysis for the Na^+-K^+-ATPase activities from all sources proceeded with temperature discontinuity points (*Td*) and two activation energies, i.e., above (Ea_1) and below (Ea_2) the breaking temperature. The calculated values for *Td* were lowest for posterior enterocytes (15.6°C) and highest for gill epithelia (22.3°C), being *Td* for pyloric caeca (17.2°C) and anterior intestine (16.4°C) enterocytes very close each together. Regarding Ea_1 it is noticeable that values of enterocytes were similar between segments (in the range 12.33 kcal/mol – 15.58 kcal/mol), but notably lower than the value obtained in gill epithelia (17.98 kcal/mol). Conversely, Ea_2 were similar between epithelial cells from intestine and gills (in the range 5.46 kcal/mol–7.72 kcal/mol). Of note, it can also be observed that maximal activities are attained in the range 30–35°C, well beyond the standard rearing (and environmental) temperature of this species, and that increasing the assay temperature above 45°C (I_t, inactivation temperature) leads to a pronounced decrease in ATPase activity, which has been interpreted as result of progressive enzyme denaturation and/or destabilization of membrane microenvironment as thermal stress increases. This thermosensitive behavior of Na^+-K^+-ATPase is common between ectotherms (Else and Wu, 1999).

By applying the transition states theory using Eyring plots we estimated activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$). Results are illustrated in the insets of **Figure 2**. From the slopes and intercepts of ln(k/T) vs. 10^3/T plots below and above *Td*, it was calculated the activation enthalpy and entropy values. In general, lower enthalpy and entropy values are observed above *Td*. In enterocytes, $\Delta H^{\ddagger}$ values were similar between sections and ranged between 11.78 kcal/mol and 12.50 kcal/ mol below *Td*,

TABLE 3 | Effects of experimental diets on lipid classes and total polar lipid fatty acid composition of isolated epithelia.

	Pyloric Caeca		Anterior Intestine		Posterior Intestine		Gill epithelia	
	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient
LIPID CLASSES								
LPC	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.86 ± 0.12	0.68 ± 0.29*
SM	1.18 ± 0.36	1.08 ± 1.01	0.67 ± 0.06	0.61 ± 0.14	0.80 ± 0.04	0.41 ± 0.21*	1.52 ± 0.29	1.02 ± 0.38
PC	16.21 ± 5.36	10.31 ± 2.75	16.61 ± 0.10	11.30 ± 1.89*	13.61 ± 3.50	11.40 ± 3.23	8.23 ± 0.70	8.71 ± 3.06
PS	2.12 ± 0.25	1.58 ± 0.31	2.24 ± 0.49	2.04 ± 0.10	3.07 ± 1.00	2.04 ± 0.34	3.16 ± 0.31	3.79 ± 0.38
PI	4.50 ± 1.18	3.32 ± 0.70	3.82 ± 0.12	2.85 ± 0.73	3.28 ± 0.64	3.36 ± 0.61	2.49 ± 0.24	2.13 ± 0.41
PG	5.06 ± 0.51	3.89 ± 0.67	4.59 ± 0.02	3.49 ± 1.05	2.27 ± 1.64	3.29 ± 0.65	2.54 ± 0.112	2.44 ± 0.23
PE	12.14 ± 1.57	7.43 ± 1.04*	13.12 ± 0.30	10.97 ± 1.93	12.39 ± 2.51	13.30 ± 1.42	6.81 ± 0.43	6.11 ± 1.80
TPL	41.22 ± 8.18	27.63 ± 4.20*	41.07 ± 0.21	31.28 ± 5.21*	35.44 ± 7.40	33.84 ± 5.02	26.64 ± 0.46	24.88 ± 5.57
MAG	1.10 ± 0.82	1.32 ± 1.01	0.56 ± 0.05	0.15 ± 0.11*	1.05 ± 0.43	0.69 ± 0.08	1.33 ± 0.44	1.38 ± 2.23
DAG	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.34	0.28 ± 0.66
CHO	28.90 ± 3.38	28.25 ± 3.18	21.37 ± 0.53	20.77 ± 1.62	26.46 ± 1.72	28.45 ± 0.84	28.52 ± 1.02	27.38 ± 3.78
FFA	8.72 ± 5.87	5.26 ± 2.75	12.66 ± 1.35	10.20 ± 3.03	17.25 ± 8.64	9.22 ± 1.23	10.21 ± 0.79	10.72 ± 3.20
TAG	17.18 ± 5.57	31.89 ± 5.62*	21.06 ± 1.45	33.51 ± 3.00*	16.64 ± 2.21	23.79 ± 4.27*	21.78 ± 1.33	21.07 ± 3.65
SE	2.85 ± 0.08	5.62 ± 0.93*	2.44 ± 0.13	4.03 ± 0.83*	2.91 ± 1.41	3.82 ± 0.67	11.13 ± 0.31	11.29 ± 0.70
TNL	58.77 ± 8.18	72.36 ± 4.20*	58.10 ± 0.63	68.71 ± 5.21*	65.04 ± 7.29	65.98 ± 5.03	73.35 ± 0.46	72.12 ± 5.57
PHO/CHO	1.43	0.98	1.92	1.51	1.34	1.19	0.87	0.88
LT (μg/mgprot)	767 ± 90	1090 ± 65*	865 ± 60	1230 ± 180*	761 ± 90	998 ± 190*	520 ± 20	518 ± 70
FATTY ACIDS								
14:0	0.42 ± 0.12	0.60 ± 0.24	1.45 ± 1.45	0.44 ± 0.28	0.59 ± 0.08	0.41 ± 0.20	0.96 ± 0.39	0.68 ± 0.23
16:0	16.88 ± 0.55	14.59 ± 2.57	18.61 ± 5.12	12.10 ± 2.68	17.36 ± 1.19	11.63 ± 0.67*	22.85 ± 3.92	22.65 ± 5.23
16:1	1.60 ± 0.32	1.78 ± 0.24	2.19 ± 1.03	1.19 ± 0.48	1.61 ± 0.23	1.18 ± 0.23	3.76 ± 0.50	2.19 ± 0.16*
18:0	11.22 ± 1.25	9.88 ± 1.80	13.11 ± 0.64	10.63 ± 2.72	13.47 ± 1.18	10.15 ± 1.69*	11.81 ± 2.01	12.11 ± 4.14
18:1n-9	9.07 ± 2.85	21.41 ± 6.72*	9.98 ± 3.34	23.84 ± 9.39*	6.86 ± 1.52	22.92 ± 5.35*	14.29 ± 3.22	20.51 ± 4.55
18:1n-7	2.73 ± 0.17	2.71 ± 0.10	2.76 ± 0.11	2.72 ± 0.20	2.31 ± 0.22	2.60 ± 0.20	3.06 ± 0.19	2.74 ± 0.30
18:1n-5	0.10 ± 0.09	0.11 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.06	0.06 ± 0.11	0.00 ± 0.00	0.09 ± 0.16
18:2n-6	5.29 ± 0.28	7.43 ± 1.97	4.87 ± 0.78	8.32 ± 3.42	4.95 ± 0.68	7.37 ± 1.50	5.81 ± 0.58	6.87 ± 2.92
18:3n-3	0.32 ± 0.06	0.24 ± 0.04*	0.44 ± 0.07	0.34 ± 0.05*	0.30 ± 0.08	0.27 ± 0.07	0.38 ± 0.04	0.11 ± 0.18*
18:4n-3	0.16 ± 0.08	0.20 ± 0.17	0.17 ± 0.07	0.10 ± 0.11	0.07 ± 0.06	0.14 ± 0.02	0.06 ± 0.11	0.05 ± 0.09
20:1	0.72 ± 0.06	1.05 ± 0.17	0.99 ± 0.13	0.92 ± 0.80	0.77 ± 0.06	1.52 ± 0.28*	1.47 ± 0.65	1.23 ± 0.24
20:2n-6	0.47 ± 0.04	0.53 ± 0.14	0.43 ± 0.09	0.58 ± 0.24	0.20 ± 0.34	0.28 ± 0.49	0.49 ± 0.07	0.64 ± 0.16
20:4n-6	2.68 ± 0.48	1.33 ± 1.18	1.56 ± 1.36	1.48 ± 0.42	2.16 ± 0.15	1.52 ± 0.21*	2.11 ± 0.96	1.78 ± 0.62
20:4n-3	0.63 ± 0.04	0.54 ± 0.16	0.60 ± 0.26	0.50 ± 0.24	0.56 ± 0.18	0.25 ± 0.26	0.10 ± 0.17	0.08 ± 0.13
20:5n-3	7.71 ± 0.65	6.10 ± 3.00	6.62 ± 2.28	5.92 ± 2.75	4.84 ± 1.56	4.37 ± 1.96	2.88 ± 1.08	1.37 ± 1.20
22:1	0.05 ± 0.04	0.10 ± 0.09	0.09 ± 0.09	0.14 ± 0.14	0.02 ± 0.04	0.05 ± 0.08	0.41 ± 0.72	0.00 ± 0.00
22:5n-6	0.70 ± 0.05	0.46 ± 0.15	0.79 ± 0.11	0.68 ± 0.40	0.96 ± 0.10	0.57 ± 0.22*	0.55 ± 0.14	0.24 ± 0.21*
22:5n-3	3.08 ± 0.50	2.32 ± 0.54	2.31 ± 0.74	2.26 ± 0.54	3.19 ± 0.51	2.50 ± 0.53	1.31 ± 0.32	1.23 ± 0.22
22:6n-3	27.12 ± 1.09	18.18 ± 2.10*	23.57 ± 3.39	18.17 ± 3.07*	28.83 ± 1.50	21.72 ± 2.32*	13.46 ± 4.99	11.87 ± 2.96
TOTALS AND INDEXES								
Monoenes	15.73 ± 2.42	28.17 ± 6.45*	17.31 ± 2.67	29.86 ± 9.76*	12.93 ± 0.98	29.55 ± 5.13*	25.54 ± 5.32	29.79 ± 4.23
Saturates	30.02 ± 1.25	26.56 ± 4.66	35.17 ± 7.79	24.39 ± 6.12	33.11 ± 2.76	23.37 ± 2.60*	37.46 ± 6.44	36.58 ± 9.60
n-3	39.20 ± 0.45	27.74 ± 5.67*	33.88 ± 6.20	27.32 ± 6.13	38.05 ± 3.35	29.60 ± 5.06	18.19 ± 6.56	14.71 ± 3.83
n-6	9.46 ± 0.29	10.40 ± 1.24	7.81 ± 2.14	11.20 ± 3.05	8.60 ± 0.46	10.14 ± 1.54	9.16 ± 1.70	9.82 ± 3.82
n-3LCPUFA	38.61 ± 0.43	27.17 ± 5.62*	33.10 ± 6.27	26.85 ± 6.13	37.51 ± 3.31	28.92 ± 4.74	17.75 ± 6.49	14.55 ± 3.56
n-3/n-6	4.15 ± 0.17	2.73 ± 0.89	4.44 ± 0.56	2.70 ± 1.42	4.44 ± 0.51	3.02 ± 0.97	1.95 ± 0.34	1.56 ± 0.29
18:1/n-3LCPUFA	0.23 ± 0.07	0.85 ± 0.43	0.30 ± 0.08	0.98 ± 0.61	0.18 ± 0.03	0.83 ± 0.34*	0.88 ± 0.35	1.42 ± 0.19*
UI	261.62	206.09	227.69	209.64	252.62	220.28	152.06	137.98

*$p < 0.05$ compared to controls. Abbreviations as in **Table 2**.

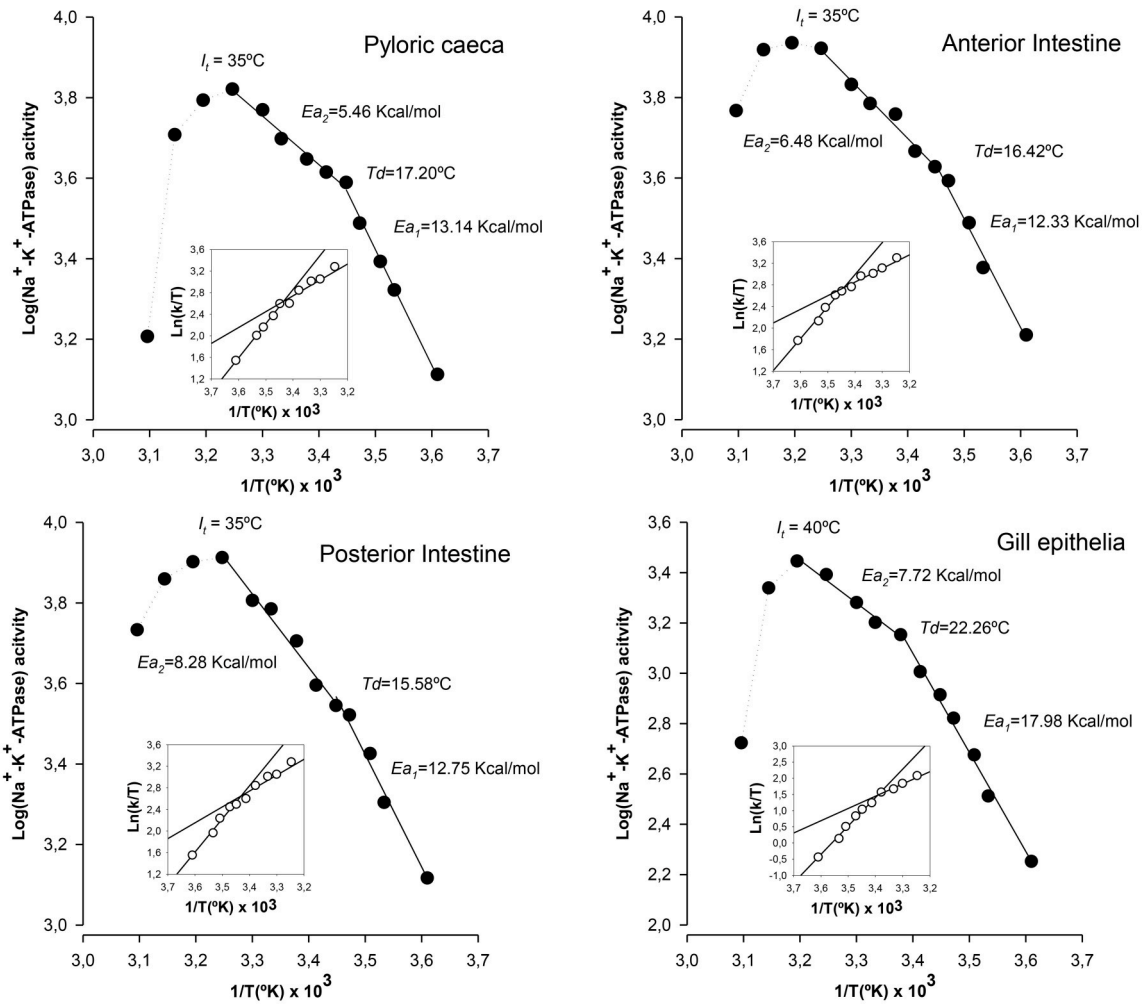

FIGURE 2 | Thermodynamic features of Na^+-K^+-ATPase from gilthead seabream epithelia reared under standard conditions. Illustrated correspond to Arrhenius plots (Log[activity] vs. 1/T) and Eyring plots (insets, Ln[k/T] vs. 1/T) for the hydrolysis of ATP by Na^+-K^+-ATPase in each epithelial preparation. *Td*: Arrhenius breaking (or discontinuity) temperature. Ea_1 and Ea_2: activation energies below and above the point of discontinuity, respectively. I_t: discontinuity at inactivation temperature. Each point corresponds to the average of four different experiments.

and between 4.92 kcal/mol and 7.77 kcal/mol above *Td*. These values were considerably higher for the case of gill epithelia, which were 18.53 kcal/mol and 10.06 kcal/mol as calculated below and above *Td*, respectively. Regarding activation entropy, highest entropy values were observed for gill epithelia both above (36.86 kcal/°K/mol) and below (65.95 kcal/°K/mol) *Td*, while in enterocytes, $\Delta S^{\ddagger}$ remained similar and ranging between 48.49 kcal/°K/mol (caeca) and 46.01 kcal/°K/mol (anterior intestine) below *Td*, and 31.78 kcal/°K/mol (anterior intestine) and 22.07 kcal/°K/mol (caeca) above *Td*.

Effects of Alterations in Lipid Profiles on the Thermodynamic Properties of Na^+-K^+-ATPase

We next assessed the potential effects of lipid alterations induced by n-3 PUFA deficient diets on the thermodynamic properties of Na^+-K^+-ATPase from intestinal and branchial epithelial cells. The results are illustrated in **Figure 3**. The first obvious differences when compared to control cells were the disappearance of *Td* and the increase in activation energy values in the enzyme from enterocytes. Indeed, Ea_1 values ranged from 50.13 kcal/mol in caecal enterocytes to 24.9 kcal/mol in the case of anterior enterocytes. The second most dramatic change was the increased thermosensitivity displayed by the enzyme from enterocytes, which started to inactivate (I_t) around 26°C, nearly 20° lower than in control cells. Consequently, Ea_2 could not be estimated in enterocyte populations. These deleterious effects of n-3 PUFA deficiency in enterocytes are indicative of membrane instability and protein inactivation as a consequence of environment destabilization. Strikingly, gill epithelium demonstrates, once again, a considerable resistance to LCPUFA depletion and its effects were much less prominent in *Td*, Ea_1, and Ea_2, though slight differences compared

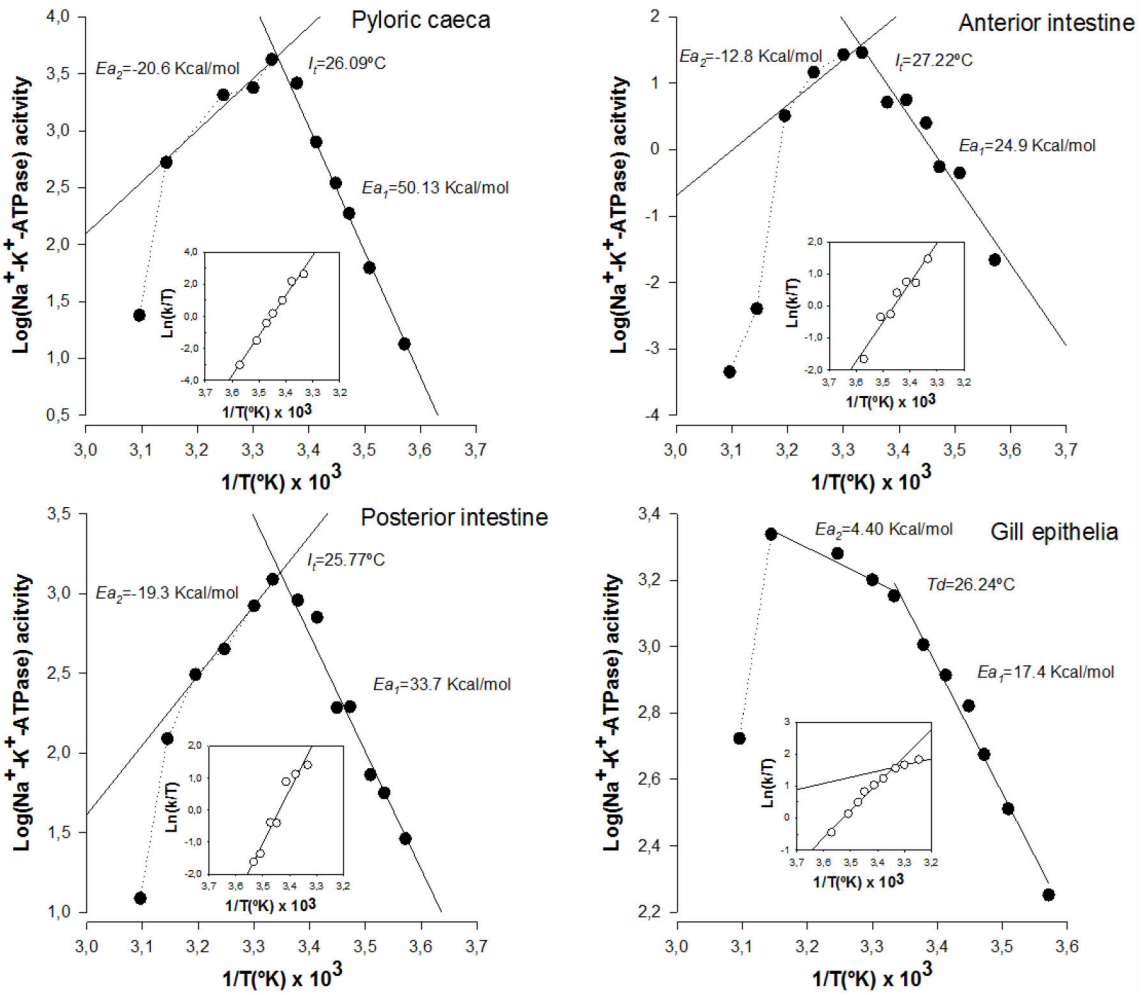

FIGURE 3 | Effects of PUFA-deficient diets on thermodynamic properties of Na^+-K^+-ATPase from gilthead seabream enterocytes from anterior intestine, posterior intestine, pyloric caeca, as well as from gill epithelium. Illustrated correspond to Arrhenius and Eyring plots for the hydrolysis of ATP by Na^+-K^+-ATPase in each epithelial preparation as indicated in **Figure 2**. Each point corresponds to the average of four different experiments.

to control animals were detected for Td and Ea_2, but not for Ea_1.

Changes in activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) for Na^+-K^+-ATPase in enterocytes below (I_t) were dramatic, with a generalized increase in $\Delta H^{\ddagger}$ (24.36 kcal/mol, 33.14 kcal/mol and 44.61 kcal/mol in anterior, posterior and caecal enterocytes, respectively) and $\Delta S^{\ddagger}$ (84.26 kcal/°K/mol, 113.94 kcal/°K/mol and 171.27 kcal/°K/mol in anterior, posterior and caecal enterocytes, respectively). However, for gill epithelia, $\Delta H^{\ddagger}$ values remained similar to controls both above (10.06 kcal/mol) and below (18.53 kcal/mol) Td. Likewise, $\Delta S^{\ddagger}$ changes closely resembled those of controls with values of 36.68 kcal/°K/mol and 65.95 kcal/°K/mol, above and below discontinuity point, respectively.

As n-3 LCPUFA deficiency affected only fatty acid composition of membrane phospholipids, but not lipid classes, levels of most relevant fatty acids were compared with the Arrhenius plot parameters (Td, Ea_1, Ea_2) and activation enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) for enterocytes belonging to the three regions as well as for gill epithelia (**Figure 4**). Initially, we obtained the Pearson's correlation coefficient matrixes for all fatty acids and ratios which were differentially affected by diets against thermodynamic parameters. First, we found significant relationships between levels of 18:1n-9 ($Td = 9.46 + 0.83$*[18:1n-9], $r = 0.96$, $p < 0.001$), total monoenes ($Td = 6.76 + 0.63$*[monoenes], $r = 0.96$, $p < 0.001$), DHA ($Td = 32.72 - 0.61$*[DHA], $r = 0.93$, $p < 0.05$) or n-3 LCPUFA ($Td = 3.04 - 0.38$*[n-3 LCPUFA], $r = 0.91$, $p < 0.05$), and Td (**Figure 4A**, left panel). Noticeably, it was observed an opposite influence of monoenes (positively related) and n-3 polyunsaturated fatty acids (negatively related) and Td. The positive effect of monoenes was also related, on one side, to saturated fatty acids as revealed by the negative relationships between saturates/18:1n-9 ($Td = 34.48 - 8.012$*[saturates/18:1n-9], $r = 0.85$, $p < 0.05$) and

Td, and to n-3 LCPUFA, on the other side, as indicated by the negative correlation between n-3 LCPUFA/monoenes ($Td = 26.30 - 3.93$*[n-3 LCPUFA/monoenes], $r = 0.88$ $p < 0.05$) and *Td* (**Figure 4A**, middle panel). More precisely, this later relationship could be defined as a very significant negative exponential relationship between the ratio DHA/18:1n-9 ($Td = 14.69 + 20.66*e^{-0.81*[DHA/18:1n-9]}$, $r = 0.96$, $p < 0.005$) and *Td* (**Figure 4A**, right panel). Since *Td* disappeared in n-3 LCPUFA deficient enterocytes (but not in gills) these epithelia were excluded from these regression analyses.

On the other hand, Ea_1 was only related to total monoenes and particularly to 18:1n-9 ($Ea_1 = 14.43 + 0.086*[1.2^{[18:1n-9]}]$, $r = 0.91$, $p < 0.005$) levels, but not obvious relationships were observed for saturated or polyunsaturated fatty acids individually (**Figure 4B**, left panel). However, these later two lipid groups appear to substantially affect Ea_1, since their ratios to 18:1n-9 provided good estimations of Ea_1 (**Figure 4B**, right panel), revealing in both cases very significant exponential decays.

Finally, we assessed the relationships between activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) as dependent variables and lipid parameters. We found that enthalpy of activation was closely related to the DHA/18:1n-9 ratio ($\Delta H^{\ddagger} = 13.17 - 198.7*e^{-3.17*[DHA/18:1n-9]}$, $r = 0.92$, $p < 0.01$) and to saturates/18:1n-9 ($\Delta H^{\ddagger} = 11.43–94.57*e^{-2.11*[saturates/18:1n-9]}$, $r = 0.98$, $p < 0.001$) (**Figure 4C**, left panel). Enthalpy ($\Delta H^{\ddagger}$) of activation was also positively (and exponentially) related to monoenes, in particular to 18:1n-9 ($\Delta H^{\ddagger} = 11.63 + 0.28*e^{-0.17*[/18:1n-9]}$, $r = 0.96$, $p < 0.005$) but not to n-3 LCPUFA or DHA (**Figure 4C**, middle panel). In the case of activation entropy ($\Delta S^{\ddagger}$), two relevant relationships were observed, i.e., for 18:1n-9 ($\Delta S^{\ddagger} = 46.22 - 0.57*e^{-0.18*[18:1n-9]}$, $r = 0.96$, $p < 0.005$) (shown in **Figure 4C**, right panel), and for the ratio DHA/18:1n-9 ratio ($\Delta S^{\ddagger} = 50.38 + 778.31*e^{-3.46*[DHA/18:1n-9]}$, $r = 0.92$, $p < 0.01$) (not shown).

DISCUSSION

In the present study, we have assessed the potential involvement of membrane lipid profiles and thermodynamic properties of the Na^+-K^+-ATPase in four populations of epithelial cells in the gilthead seabream. First, we observed that fatty acid composition of membrane phospholipids slightly differ between enterocytes isolated from the different intestinal segments. However, most dramatic differences were observed between enterocytes and branchial epithelial cells. To the best of our knowledge this is the first study convincingly demonstrating such heterogeneities in membrane lipid composition of epithelial cells. Highlighting main differences, we observed that, under control conditions, enterocytes are more enriched in long-chain polyunsaturated fatty acids (mainly DHA), total polar lipids, PC, PE, PS, PI, and PG, compared to gill epithelial cells. Conversely, branchial cells contain higher levels of total neutral lipids (mainly CHO, FFA, MAG, and TAG), and 20-carbons polyunsaturated fatty acids (ARA and EPA), as well as monoenes. These features allowed a neat discrimination between epithelial cells in the multivariate analyses performed here, which allowed us to define a differential lipid fingerprint between epithelial cells of intestinal and branchial origins. As the biochemical structure of membrane bilayer is finely tuned to cope with the specific cellular functions, it can be speculated that in gill epithelia, membrane phospholipids are more adequate to provide an efficient permeability transmembrane barrier against severe osmo-ionic gradients (Wood and Shuttleworth, 1995; Evans et al., 2005). Another conclusion that can be extrapolated from these data, is that as membrane surface is greater in enterocytes than in branchial cells (due to the presence of an extensive brush border), it would be expected a higher level of TPL. On the other hand, epithelial branchial cells exhibit higher levels of FFA, MAG, and TAG, which is in consonance with the high density of mitochondria and higher metabolic rates, especially in chloride cells (Wood and Shuttleworth, 1995; Evans et al., 2005), which accomplish the major task of NaCl extrusion against a very large electrochemical gradient.

The second goal of our study was the assessment of the extent to which lipid profiles in epithelial cells might be affected by dietary modifications. Our dietary manipulation consisted in providing an n-3 PUFA-deficient diet which contained similar amount of total lipids compared to control standard diets, but changed proportions of PUFA, monoenoic and saturated fatty acids. The results showed that deficient diets have a profound impact on lipid profiles in enterocytes. With the sole exception of TAG which were significantly increased in all intestinal segments studied, lipid classes remained unaffected. However, most important changes were observed in fatty acid composition of membrane phospholipids. Thus, levels of total monoenes (mainly 18:1n-9) were significantly increased, while DHA, total n-3 LCPUFA and 18:3n-3, were reduced. All these findings revealed a direct influence of diet composition on the enterocyte membrane composition. However, it is worthwhile mentioning that the increase in 18:1n-9 in membrane phospholipids may also be part of a homeoviscous strategy to keep membrane fluidity in conditions of LCPUFA depletion. Strikingly, fatty acid composition of branchial cells from the same animals remained very similar between control and deficient diets, with only small changes in some minor fatty acids. These observations are very relevant from the homeostatic point of view, and pinpoints to the existence of powerful lipostatic mechanisms responsible for the biochemical stability of branchial cell membranes (including chloride cells, mucous cells, epithelial respiratory cells and pillar cells). This indeed leads us to hypothesize that the extremely relevant physiological functions of branchial cells (Wood and Shuttleworth, 1995; Evans et al., 2005) are tightly linked to a precise lipid composition in cell membranes.

The next goal in the present study was the assessment of how changes in lipid profiles of cell membranes might affect the thermodynamic properties of epithelial Na^+-K^+-ATPase. Results from Arrhenius plots indicate that the overall reactions of ATP hydrolysis for the Na^+-K^+-ATPase activities from all sources followed convex behaviors (Truhlar and Kohen, 2001) with temperature discontinuity points (*Td*) and two activation energies, above (Ea_1) and below (Ea_2) the discontinuity, with

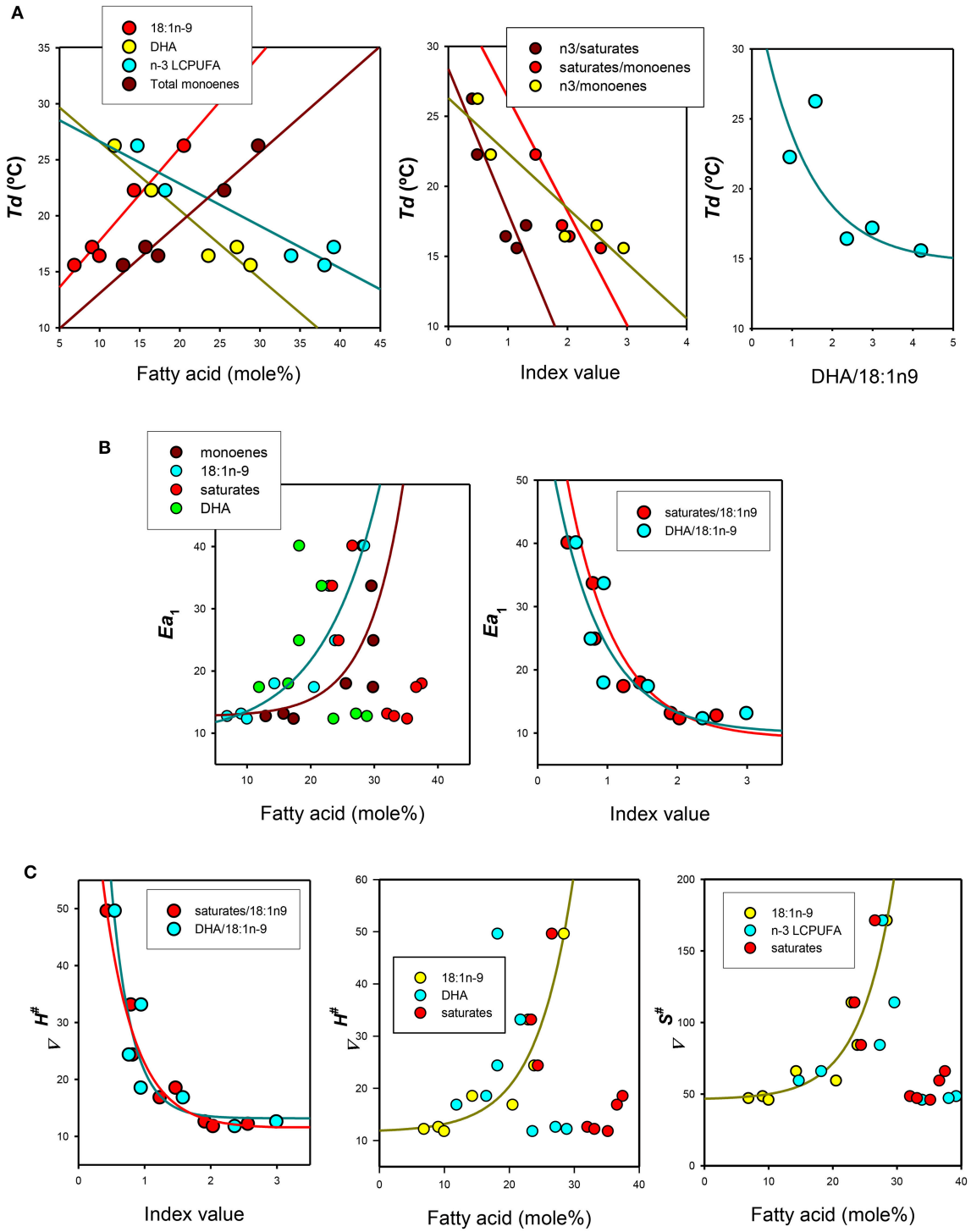

FIGURE 4 | Relationships between thermodynamic signatures of Na^+-K^+-ATPase from gilthead seabream epithelia and their lipid features. (A) Effects of individual, grouped or ratios fatty acids on Arrhenius (discontinuity) breakpoints (*Td*). **(B)** Effects of lipid parameters on activation energies below *Td or* l_t*:* (Ea_1). **(C)** Effects of lipid ratios and selected fatty acids on activation enthalpy ($\Delta H^{\ddagger}$) and activation enthalpy ($\Delta S^{\ddagger}$).

$Ea_1 > Ea_2$. Slight differences in these parameters were detected for control enterocytes, but values for control gill epithelia were substantially different, being *Td* and Ea_1 higher than in enterocytes. Similar results have been observed in mucosal scrapings (Almansa et al., 2003) and branchial microsomal fractions from gilthead seabream (Ventrella et al., 1990) and in

the sea bass *Dicentrarchus labrax* (Ventrella et al., 1993) reared under similar conditions.

Thermodynamically, the lower *Td* values obtained for enterocytes imply lower values for the thermotropic transitions of protein-lipid and protein-protein interactions and, coherently, a temperature reduction in the activation energies-shift point (Truhlar and Kohen, 2001). Accordingly, the enzyme from pyloric caeca would display lower activation energy above 17.20°C, which would allow an increase in the enzyme activity at lower temperatures (close to *Td*), compared with anterior and posterior enterocytes, whose shift points were clearly lower. Conversely, the enzyme from gills would require substantially higher temperatures (above 22.26°C) to benefit from a lower activation energy (as inferred from Ea_2). This apparent unfavorable condition may not be compensated by the $\Delta H^{\ddagger}$ values (both above and below Td) found in gill Na^+-K^+-ATPase. These assertions have been demonstrated experimentally in enzyme activity assays performed under standard conditions, where branchial Na^+-K^+-ATPase specific activity measured at 20°C was found ∼50% lower than in averaged enterocytes. Also, these results are in line with lower activation enthalpy for enterocytes compared to gill epithelia (**Table 4**). Thus, it seems that there exist environmental restrictions leading to restricted degrees of freedom (i.e., translational, rotational, and vibrational) in the transition state for the hydrolysis of ATP by Na^+-K^+-ATPase in branchial membranes. This is perhaps the price must be paid in gill epithelia to cope with extreme osmotic and ionic gradients far from equilibrium. It is known that the Na^+-K^+-ATPase reaction cycle includes two major conformational intermediates, namely *E1* (with high affinity for ATP) to *E2* (with phosphatase activity) (Schuurmans-Stekhoven and Bonting, 1981; Skou and Esmann, 1992; Kaplan, 2002), and that the transition between them occurs with a large enthalpy and entropy change. Moreover, changes in membrane order (reduction) favors the *E1* conformation and ATP binding (i.e., by PUFA or ethanol) while the increase in membrane order by reduced temperature or PUFA depletion, favors the *E2* conformation with ATP hydrolysis and K^+ binding (Swann, 1986; Skou and Esmann, 1992; Kaplan, 2002). Thus, the higher absolute values of $\Delta H^{\ddagger}$ (and $\Delta S^{\ddagger}$) of activation for the hydrolysis of ATP in gill Na^+-K^+-ATPase suggest more ordered membranes, where the stabilization forces required for the transition of *E1* to *E2* conformations for hydrolysis are higher than for enterocytes. Indeed, our analyses of membrane lipid composition (with significantly lower contents of total n-3 LCPUFA in gill epithelia) and the lower membrane unsaturation index found in gill cell membranes (194.35) compared to enterocytes' (ranging 244.4–256.7) support the thermodynamic observations and may well explain the observed differences between intestinal and branchial epithelia.

The relationships between epithelial membrane lipids and thermodynamic properties of Na^+-K^+-ATPase was further demonstrated in the n-3 PUFA deficiency experiments. This maneuver dramatically affected lipid profiles in enterocytes. As mentioned before, the main effects being a considerable increase in oleic acid and a substantial DHA depletion. Paralleling these findings, *Td* disappeared and Ea_1 increased by 2–3.8-fold compared to control animals. LCPUFA depletions also augmented the thermosensitivity of intestinal Na^+-K^+-ATPase and reduced the values for inactivation temperature (I_t). Noticeably, none of these parameters were significantly affected in gill epithelia, which reinforce the hypothesis that branchial cells are endowed with a highly efficient lipostatic mechanism ensuring the biochemical stability of membrane lipids (and lipid-lipid and lipid-protein interactions), ultimately giving support to the splendid physiological complexity of fish gills.

Severe changes in absolute values of $\Delta H^{\ddagger}$ (and $\Delta S^{\ddagger}$) of activation for the hydrolysis of ATP were observed in enterocytes (but again not in gill epithelia), which increased by 2–4-fold for $\Delta H^{\ddagger}$ and 1.8–3.5 fold for $\Delta S^{\ddagger}$ below inactivation temperature (I_t). These observations suggest more ordered membranes and higher stabilization forces for the transition *E1* to *E2* conformations. In agreement, unsaturation indexes in enterocytes (but not in branchial cells) were reduced by LCPUFA depletion by 14% (index values ranging 261-227 in controls, and 206-227 in LCPUFA deficient cells). The deleterious effects of increased Ea_1, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were readily demonstrated in the specific activity assays (as measured at 20°C, the rearing temperature of seabream). Indeed, unlike branchial preparations,

TABLE 4 | Summary of thermodynamic parameters calculated for epithelial cells from control individuals and animals fed LCPUFA deficient diets.

	Ea_1 (kcal/mol)	Ea_2 (kcal/mol)	*Td* (°C)	$\Delta H^{\ddagger}$ < *Td* (kcal/mol)	$\Delta H^{\ddagger}$ > *Td* (kcal/mol)	$\Delta S^{\ddagger}$ < *Td* (kcal/°K/mol)	$\Delta S^{\ddagger}$ > *Td* (kcal/°K/mol)
CONTROLS							
Gill	17.98	7.72	22.26	18.53	10.06	65.96	36.87
Pyloric caeca	13.14	5.46	17.20	12.59	4.92	48.50	22.07
Anterior intestine	12.33	6.48	16.42	11.78	7.00	46.01	25.87
Posterior intestine	12.75	8.28	15.58	12.20	7.77	47.13	31.78
LCPUFA DEFICIENT							
Gill	17.40	4.40	26.24	16.84	3.83	59.41	15.92
Pyloric caeca	50.13#		26.09#	49.61#		171.27#	
Anterior intestine	24.91#		27.22#	24.36#		84.26#	
Posterior intestine	33.68#		25.77#	33.14#		113.93#	

#*Values were obtained below* I_t.

in enterocytes' homogenates, Na^+-K^+-ATPase specific activity fell by average 82.8% compared to controls.

In order to explore more deeply the individual relationships between specific membrane lipids and lipid ratios, as independent variables, and thermodynamic parameters, we used non-linear regression analyses. We found that *Td* was significantly negatively related to DHA and positively to 18:1n-9 (and monoenes). Expectedly, the ratio n-3 LCPUFA/monoenes (and more precisely the ratio DHA/18:1n-9) was negatively related to both *Td*, following a negative exponential relationship. Overall, these data indicates that the relative amounts of 18:1n9 and DHA in membrane phospholipids are critical in determining the discontinuity point and the transition $Ea_1 \leftrightarrow Ea_2$ and pinpointed that small changes in DHA and/or 18:1n-9, have a great impact in setting *Td* as per their exponential relationship. Moreover, the same ratio (DHA/18:1n-9) was also found to be a significant predictor of Ea_1 and $\Delta H^{\ddagger}$ values, observing in both cases negative exponential equations describing their relationships. In these cases, both total monoenes and 18:1n-9 (but not saturates or DHA alone) were significantly related to Ea_1, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ but following exponential relationships, which indicates a very relevant role of 18:1n-9. Overall, these data indicate the relevant effects of DHA and monoenoic fatty acids (and their relative proportions) in setting thermodynamic traits for Na^+-K^+-ATPase, at least for ATP hydrolysis.

Finally, current available information based on reconstitution and crystallographic studies may provide, at least in part, a molecular interpretation in support of our observations. Thus, recent studies using purified detergent-soluble recombinant α/β or α/β/FXYD Na^+-K^+-ATPase complexes, have revealed three separate functional effects of phospholipids and cholesterol that are exerted at separate binding sites for phophatidylserine/cholesterol (site A), polyunsaturated phosphatidylethanolamine (site B), and saturated PC or sphingomyelin/cholesterol (site C) (Cornelius et al., 2015; Habeck et al., 2015). These binding sites likely correspond to three lipid-binding pockets identified in the crystal structures of the Na^+-K^+-ATPase (Shinoda et al., 2009; Laursen et al., 2013). The three sites appear to have different effects on Na^+-K^+-ATPase complexes, being site A stabilizing, site B stimulatory and site C inhibitory (Cornelius et al., 2015; Habeck et al., 2015). Therefore, it is expected that direct and specific interactions of different phospholipids and cholesterol within the protein molecular framework, will determine both the stability and molecular activity of Na^+-K^+-ATPase, eventually giving membrane lipid composition an essential role in its physiological regulation. As it has been mentioned before, the activating site is associated to polyunsaturated fatty acid binding, especially when esterifying the *sn*-2 position of neutral lipids such phosphatidylethanolamine or phosphatidylcholine. Indeed, these two phospholipids represent by average 66% of epithelial TPL (**Tables 2, 3**), and DHA is, by far, the most abundant fatty acid in epithelial polar lipids. Recently, the stimulation of Na^+-K^+-ATPase activity of the purified human α1β1 or α1β1FXYD1 complexes by neutral PUFA PC or PE has been reported, being the stimulation structurally selective for neutral phospholipids (Haviv et al., 2013). The structural selectivity for the neutral phospholipid and asymmetric saturated plus PUFA fatty acyl chain structure is actually a strong indication for a specific interaction with Na^+-K^+-ATPase. Indeed, molecular modeling of *E1∼P* and *E2·P* conformations bound to any of these polyunsaturated phospholipids suggests that their specific binding may facilitate the *E1P↔E2P* conformational transition (mainly by lowering the activation energy) (Cornelius et al., 2015). In addition, DHA (and other LCPUFA) impose a general physical effect on the bilayer physicochemical state as it has conformational properties that keep highly structured but fluid membrane bilayer capable to accommodate rapid protein conformational changes (Rabinovich and Ripatti, 1991; Stillwell and Wassall, 2003; Diaz et al., 2012). Presumably, adjustment of DHA levels within membrane phospholipids (mainly phosphoglycerides) would accomplish effective physicochemical changes over a wide temperature range, ensuring the adaptation of cell membranes to environmental fluctuations and metabolic activity (Sargent et al., 1995). The latter being considered an important mechanism involved in thermal acclimation in ectothermic organisms (Raynard and Cossins, 1991; Else and Wu, 1999; Ernst et al., 2016). Interestingly, the stimulatory effects of polyunsaturated PE or PC seem to be independent of cholesterol and the FXYD protein (Cornelius et al., 2015). This is relevant because cholesterol is quite abundant in epithelial lipid profiles (the second most abundant neutral lipid after TAG, and accounting for ∼40% of TNL), and because cholesterol has been long recognized to be essential for optimal Na^+-K^+-ATPase activity (Crockett and Hazel, 1997; Cornelius, 2001; Cornelius et al., 2015). The effects of cholesterol on Na^+-K^+-ATPase are associated to its ability to impose a high degree of conformational order on the phospholipids acyl chains thereby stabilizing the liquid-ordered lipid phase, which is dominated by changes in activation entropy (Cornelius et al., 2015), but also to direct interaction with site A (Shinoda et al., 2009; Habeck et al., 2015). However, as we observed no changes in cholesterol contents, neither between tissues nor between diets (**Table 3**), observed differences in activation $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ cannot be explained by distinct membrane cholesterol.

Finally, we wish to introduce a word of caution on the extent to which our results may be interpreted. Indeed, although we have empirically studied the thermodynamic parameters of the epithelial Na^+-K^+-ATPase from intestinal and branchial origins, and correlated them with the membrane phospholipid composition, it is not possible to provide molecular details on the real enzyme-membrane interactions nor giving precise insights into the physical mechanisms underlying the observed responses.

In conclusion, the results illustrated in the present study reveal that subtle differences in the lipid matrix of the lipid microenvironment surrounding the Na^+-K^+-ATPase may explain the heterogeneous thermodynamic features of the Na^+-K^+-ATPase in epithelia from the gilthead seabream reared under control conditions. In enterocyte preparations, these effects were exacerbated by induction of membrane n-3 LCPUFA deficiency. Noteworthy, epithelial cells from gill origin were notably resistant to diet-induced modifications of membrane lipid composition and, consequently, to alterations in the thermodynamic features

of the Na^+-K^+-ATPase. We conclude that n-3 LCPUFA and 18:1n-9 (together with cholesterol) are critical elements for the fine tuning of the Na^+-K^+-ATPase activity within the context of the epithelial plasma membrane, which underlies the essential role of the Na^+-K^+-ATPase in the physiological regulation of osmo/ionoregulatory tasks in euryhaline teleost fish.

AUTHOR CONTRIBUTIONS

RD and CR performed the lipid analyses and specific ATPase activity experiments. TG was in charge of temperature-dependence experiments. MD designed the study, analyzed the data (together with RD) and wrote the manuscript. All authors reviewed the drafted manuscript.

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Multidimensional Liquid Chromatography Coupled with Tandem Mass Spectrometry for Identification of Bioactive Fatty Acyl Derivatives

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Recognition of the contributions of lipids to cellular physiology, both as structural components of the membrane and as modulatory ligands for membrane proteins, has increased in recent years with the development of the biophysical and biochemical tools to examine these effects. Their modulatory roles in ion channels and transporters function have been extensively characterized, with the molecular mechanisms of these activities being the subject of intense scrutiny. The physiological significance of lipids in biochemistry is expanding as numerous fatty acyls are discovered to possess signaling properties. These bioactive lipids are often found in quantities of pmol/g of tissue and are co-extracted with numerous lipophilic molecules, making their detection and identification challenging. Common analytical methodologies involve chromatographic separation and mass spectrometric techniques; however, a single chromatographic step is typically ineffective due to the complexity of the biological samples. It is, therefore, essential to develop approaches that incorporate multiple dimensions of separation. Described in this manuscript are normal phase and reversed phase separation strategies for lipids that include detection of the bioactive primary fatty acid amides and N-acyl glycines via tandem mass spectrometry. Concerted utilization of these approaches are then used to separate and sensitively identify primary fatty acid amides extracted from homogenized tissue, using mouse brains as a test case.

Keywords: bioactive lipids, fatty acyls, lipid-protein interactions, multidimensional liquid chromatography, N-acyl ethanolamines, N-acyl glycines, primary fatty acid amides

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INTRODUCTION

Lipids are classically defined as hydrophobic or amphiphilic small molecules with limited solubility in aqueous solutions where they typically aggregate non-covalently. The macromolecular complexes are constituents of cellular membranes or comprise relatively inert complexes used for energy storage. In considering the former, the lipid composition of the bilayer alters its physicochemical properties, and this local environment for receptors and other membrane proteins affect their structure and function (Coskun and Simons, 2011; Laganowsky et al., 2014). Given the focus of this thematic issue, if we restrict discussion to receptors, there are numerous examples of how the structure and function of these classes of membrane proteins are affected by lipids (for overview, see Chattopadhyay and Ruysschaert, 2015). Some dramatic examples include

the radically different topologies exhibited by lactose permease as a function of phospholipid composition (Serdiuk et al., 2015), the effects of cholesterol on ion channels and other membrane proteins (Levitan et al., 2014; Korinek et al., 2015; Lange and Steck, 2016), the functional modulation of metabotropic serotonin receptors by bilayer composition (Gutierrez et al., 2016), and the lipid dependence of glucose transporters (Hresko et al., 2016). In turn, the activity of these signaling molecules on the local membrane potential affects the nanoscale organization of the neighboring lipids, potentially affecting cellular signaling properties as shown for K-Ras signaling (Zhou et al., 2015).

In addition to these indirect mutual effects of lipids and signaling, many lipids exert bioactive properties directly as cellular signals and 2nd messengers (for reviews, see Hannun and Obeid, 2008; García-Morales et al., 2015; Morales-Lázaro and Rosenbaum, 2015). In this study, we have focused attention on fatty acyls (for review see Divito and Cascio, 2013). N-acyl ethanolamines (NAEs) have a long history of physiological and G-protein coupled receptor mediated effects that are directly related to, or reminiscent of, cannabinoid activation (Lambert et al., 2002; Ahn et al., 2008; Luchicchi and Pistis, 2012; Mechoulam and Parker, 2013). Primary fatty acid amides (PFAMs) and N-acyl glycines (NAGs), however, have been more recently recognized as bioactive signaling lipids that have demonstrated interactions with serotonin receptors (Huidobro-Toro and Harris, 1996; Huidobro-Toro et al., 1996; Thomas et al., 1997, 1999), gap junction proteins and calcium signaling (Guan et al., 1997; Boger et al., 1998; Huang and Jan, 2001; Lo et al., 2001; Rimmerman et al., 2008), and a myriad of physiological effects (Wakamatsu et al., 1990; Lerner et al., 1994; Cravatt et al., 1995; Mitchell et al., 1996; Basile et al., 1999; Fedorova et al., 2001; Huitrón-Reséndiz et al., 2001; Laposky et al., 2001; Mendelson and Basile, 2001; Murillo-Rodríguez et al., 2001; Stewart et al., 2002). Of these documented effects, the most potent bioactive lipids consist of 16–22 chain length acyl tails and can be saturated or unsaturated species. It is hypothesized that aberrant endogenous levels of these species, either by down-stream modulation or alteration of their metabolic enzymes, could contribute to disease states. This is an intriguing consideration as PFAMs have high affinity for serotonin receptors (Huidobro-Toro and Harris, 1996; Huidobro-Toro et al., 1996; Thomas et al., 1997; Lambert et al., 2002), though no reports exist for PFAM levels in a depressive phenotype. Evidence for up-regulation of NAEs exists in studies of chronic pain (Ghafouri et al., 2013), schizophrenia (Leweke et al., 1999), and depression and anxiety (Dlugos et al., 2012), yet the physiological relevance of these observations is still unclear. In light of this, it is of substantial importance to develop analysis strategies to sensitively detect and accurately quantitate bioactive fatty acyls. These methods, in combination with phenotype studies, will aid in the elucidation of the role of signaling lipids in physiology and help define their role or diagnostic value in health and disease. The generated methodologies may provide additional tools for diagnoses and development of treatment strategies.

Lipid milieus of biological samples are often very complex mixtures. Even the most common lipid extraction methods, such as Folch-Pi (Folch et al., 1957), remove a mélange of several different lipid classes. These include fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols, PFAMs, NAGs, and NAEs. Identification of isomers and isobars by standard separation methods, such as normal phase or reversed phase chromatography, is often insufficient for complete identification of all constituents.

Multidimensional liquid chromatography (MDLC) analysis is the process of separating samples with at least, but most typically, two different chromatographic separation schemes (columns or dimensions)(Giddings, 1984; Bushey and Jorgenson, 1990; Dixon et al., 2006; Cohen, 2008; Dugo et al., 2008). The main advantage of MDLC is the dramatic increase in peak capacity known as the "product rule"; where the maximum peak capacity becomes the product of the individual chromatographic peak capacities. These different separation schemes should be orthogonal, or operate by different separation mechanisms, in order to achieve maximum discrimination and capacity. Analyses by two or more different chromatographic dimensions allows for the separation of difficult to resolve components, or samples with a high number of constituents. The wide and complex lipid composition in biological samples require MDLC methodologies to sufficiently separate the lipids prior to MS or MS/MS characterization (as an additional complication may arise due to isobaric lipids). Lipid separations have been demonstrated with normal phase, reversed phase and silver ion chromatography.

Fortuitously, straight chain fatty acyls have a well-predicted elution order by reversed phase chromatography where retention is directly proportional to the length of the acyl chain. The degrees of unsaturation reduce the retention such that it is similar to acyl chains 2 carbons shorter per each degree of unsaturation. For example, a C18:1 fatty acyl would have an elution order closer to a C16:0 fatty acyl; this is known as a critical pair (Gutnikov, 1995). This is true regardless of the head group moiety. Therefore, the methods described herein could be applied to any fatty acyl subclass with adjustment to the gradient elution program.

Normal phase separations of fatty acyls have been achieved with thin layer chromatography and adapted to solid phase extraction columns (Sultana and Johnson, 2006). These methods are capable of isolating cholesterol esters, fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols, PFAMs, NAGs, and NAEs obtained from samples or sample extracts. Analyses of lipids have special process contamination and trace analysis aspects to consider. Several PFAMs and NAEs contaminants have been identified in different grades of solvents and as slip additives in plastics (Cooper and Tice, 1995; Skonberg et al., 2010). It is essential to test solvent background and to avoid plastics in all sample preparations. This usually requires the manual packing of solid phase extraction (SPE) columns, since commercial SPE cartridges are typically housed in plastic jackets. Additionally, lipids can adhere to glass surfaces; therefore, silinization of all glassware may be necessary to limit adherence and increase recovery of the analytes and/or increase their limit of detection.

Silver ion, or argenation, chromatography has been used extensively for separation of lipid samples with a high variation of unsaturation numbers and geometrical configuration, such as are commonly found in triacylglycerols (Dobson et al.,

1995; Christie, 1998a,b; Momchilova et al., 1998; Nikolova-Damyanova et al., 2003; Adlof, 2004, 2007; Dugo et al., 2004, 2006a,b; Mondello et al., 2005; Christie et al., 2007). The general mechanism of action in this separation scheme is unclear, though Christie and co-workers propose a theory of silver ions forming weak, reversible charge transfer complexes with the analytes (Dobson et al., 1995; Christie, 1998c; Momchilova and Nikolova-Damyanova, 2003; Nikolova-Damyanova, 2009). Another possibility is an intermolecular ion-dipole interaction between the immobilized silver ions on the chromatographic bed and the π bonds of the analyte. In general, elution order of analytes by this technique is saturated, trans-unsaturated, mono-unsaturated, and so on. A number of experimental parameters and analyte variables have been explored in relation to retention factor allowing implementation of this methodology in a predictable manner (Christie, 1998b; Shan and Wilson, 2002; Harfmann et al., 2008). One exception to typical chromatographic separations is that retention of analytes in silver ion chromatography is reduced at lower temperature (Adlof and List, 2004).

Multidimensional liquid chromatography (MDLC) separations have been demonstrated for triacylglycerols in plant and animal samples (Dugo et al., 2004, 2006a,b; Mondello et al., 2005). Each approach utilized a reversed phase separation with an isopropanol/acetonitrile gradient and an isocratic silver ion separation with 0.5–0.7% acetonitrile in hexane. Samples that were separated with an inline MDLC approach had a reversed phase 2nd dimension separation with a total analysis time of less than 2 min (Mondello et al., 2005; Dugo et al., 2006a). More recently, the development of hybrid mass spectrometers with front-end mobility cells for ion mobility spectrometry capabilities has provided another powerful analytical tool for lipidomic studies that may be combined with chromatographic separations to effect 3D separations (Paglia et al., 2015).

To our knowledge, MDLC methods have not yet been published for PFAMs or NAGs, although our laboratory has utilized an SPE method prior to GC-MS analyses (Sultana and Johnson, 2006). In this report we provide effective separation methods developed for the bioactive PFAM and NAG lipid standards. Separations were effected by normal phase chromatography to differentiate lipid classes, and the peaks corresponding to PFAMs and NAGs were then further separated into their component species by reversed phase chromatography. PFAMs and NAGs were then characterized by MS/MS using either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI), respectively. Given our laboratories interest in the effects of PFAMs on serotonergic systems, we then show the effectiveness of these general methods to isolate and identify PFAMs in brain tissue.

MATERIALS AND METHODS

Chemicals

Methanol (Optima grade), formic acid (Optima grade), ammonium acetate, hexane, acetonitrile, and ammonium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Oxalyl chloride, oleic acid, erucic acid, petroselaidic acid, heptane HPLC grade, methyl-tert-butyl-ether HPLC grade, isopropanol HPLC grade, acetic acid, and anhydrous dichloromethane were from Sigma Aldrich (St Louis, MO, USA). N, N-dimethylformamide, heptadecanoic acid, and eicosanoic acid were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Lauric acid, myristic acid, palmitic acid, stearic acid, and docosanoic acid were purchased from Acros Organics (New Jersey, USA). Elaidic acid was from MP Biomedical Inc. (Solon, OH, USA) and linoleamide was purchased from Enzo Life Sciences (Ann Arbor, MI, USA). Stearoyl ethanolamine, oleoylglycine, linoleoylglycine, palmitoylglycine, arachidonoylglycine, and arachidoylglycine were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 1 monopalmitoyl-rac-glycerol (MAG) and tristearin (TAG) were from Sigma (St. Louis, MO) 99% purity and 1,2-dipalmitoyl-rac-glycerol (DAG) was from MP Biomedicals (Solon, Ohio).

Normal Phase Separation

PFAM and NAG standards were prepared in a mixture at 1 mM concentration of each standard. The mixture was separated via normal phase chromatography utilizing a YMC PVA-Sil column (4.6 × 250 mm, 5 μm particle size). Gradient elution is carried out starting at 95% mobile phase A (heptane with 0.5% v/v methyl-tert-butyl-ether) and increasing linearly to 50% mobile phase B (methyl-tert-butyl-ether with 10% v/v 2-propanol and 0.2% v/v acetic acid) over 40 min with a flow rate of 1 mL/minute. Fractions were collected at 1 min intervals with an injection volume of 200 μL and the times corresponding to NAG and PFAM elution were determined by reversed phase chromatography and MS/MS detection.

Reversed Phase Separation of N-acyl Glycines

Palmitoylglycine, linoleoylglycine, oleoylglycine, stearidonoylglycine, arachidonoylglycine, and arachidoylglycine were analyzed on an Agilent Technologies 1200 Liquid Chromatography system with a 6460 Triple Quadrupole Mass Spectrometry Detector. Mobile phase A was methanol and mobile phase B water, with 10 mM ammonium acetate in both phases. Separations were carried out on a YMC Cartenoid column (4.6 × 150 mm, 5 μm particle size) with a linear gradient of 90 to 100% mobile phase A over 15 min with a 15 min hold time. An additional separation method was developed on a Phenomenex C18 column (4.6 × 100 mm, 2.6 μm particle size) with a linear gradient of 80 to 100% mobile phase A over 5 min and a 2 min hold time. A second gradient method was used and consisted of 70% mobile phase A hold for 7 min, a linear gradient increase to 80% for 7 min, a 1 min hold at 80% before increasing to 100% mobile phase A over 5 min, and a final 5 min hold for a total analysis time of 25 min.

Ionization was achieved with an ESI source operated in negative mode with optimized parameters: fragmentor voltage 135 V, sheath gas flow 11 L/minute, nebulizer pressure 55 psi, nozzle voltage 500 V, capillary voltage 3500 V, drying gas flow 9 L/minute, drying gas temperature 275°C, and dwell time of 500 ms. Multiple reaction monitoring parameters were set-up

to analyze the $[M–H]^-$ parent ions and 74 *m/z* product ion representing the glycine head group fragment.

Reversed Phase Separation of Primary Fatty Acid Amides

All primary fatty acid amide standards were synthesized in house at greater than 98% purity. Briefly, PFAMs were synthesized by a modified procedure described by Philbrook (1954) Fatty acids were dissolved in anhydrous dichloromethane and converted to the acid chloride by reaction with oxalyl chloride under anhydrous argon atmosphere. Dichloromethane solvent was removed by rotary evaporation *in vacuo*, and the remaining acid chloride was subjected to ammonia gas by inserting ammonium hydroxide filled syringes into the sealed, argon filled reaction vessel. Reactions were considered to be complete when the fatty acid chloride oil was completely converted to a white solid fatty acid amide product by visual inspection. Products were purified by liquid phase extraction with chloroform.

Purity of all synthesized amides was verified by GCMS on a Varian CP-3800 GC with Varian Saturn 2000 Ion Trap Mass Spectrometer. Gas chromatography was performed on a Varian Factor Four Capillary Column (VF-5 ms, 30 m × 0.25 mm ID) with a flow of 1 mL/min helium carrier gas. Injector temperature was held at 250°C with split injection (ratio 10). Temperature gradient started at 55°C and ramped 40°C/min to 150°C with a hold of 3.62 min before ramping 10°C/min to 275°C and holding 6.50 min. The total run time was 25 min. Eluted fatty amides were ionized by chemical ionization with methanol and analyzed in selected ion mode. The peak area of fatty acid substrate and PFAM product from GC-MS runs were used to determine purity. All PFAMs were found to be of 98% purity or greater. Lauramide (C12:0), myristamide (C14:0), linoleamide ($C18:2^{9,12}$), palmitamide (C16:0), oleamide ($C18:1^{9}$), elaidamide ($C18:1^{9trans}$), petroselaidamide ($C18:1^{6trans}$), heptadecanoamide (C17:0), stearamide (C18:0), arachidamide (C20:0), erucamide ($C22:1^{13}$), and behenamide (C22:0) were separated on a Agilent RP C18 column (2.0 × 50 mm, 1.8 μm particle size) with a gradient elution of methanol and water, both containing 0.3% formic acid.

PFAMs were detected using an Agilent 6460 Triple Quadrupole Mass Spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. Optimized detection parameters are as follows: gas temperature 325°C, vaporization temperature 325°C, gas flow 4 L/minute, nebulizer pressure 22 psi, capillary voltage 3500 V, corona 4 μA, and fragmentor 125 V. Multiple reaction monitoring was used to detected the $[M+H]^+$ parent ions and product ions of 55 and 43 *m/z* representing a short acyl chain and short alkene fragments, as determined from fragmentation studies for the monounsaturated and saturated compounds, respectively.

Extraction of Primary Fatty Acid Amides from Biological Samples

Extraction of polar lipids from biological samples was achieved by a modified Folch-Pi method (Folch et al., 1957). All samples were extracted in glassware thoroughly cleaned by soap and water, distiller water rinse, 1 M sodium hydroxide solution soak for 1 h, distilled water rinse, acetone rinse, and toluene rinse. The dried glassware was silanized with trimethylchlorosilane (Seed, 2001). All plastic materials, including pipet tips, were avoided during all steps of analysis as PFAMs are common slip additives in plastic production (Cooper and Tice, 1995).

Samples of mouse brain (a generous gift from Dr. S. Amara, Univ. of Pittsburgh School of Medicine) were weighed and frozen at 20°C. The tissue sample was homogenized in a 2:1 chloroform:methanol solvent mixture containing 1 mM indomethancin with a volume of 20 times the sample weight. Heptadecanoamide was added as an internal standard to a final concentration of 10 μM. The insoluble material was removed by centrifugation and the supernatant was vortexed with an aqueous 10% KCl solution to remove salts, proteins, and water soluble components. The organic phase was dried under a stream of nitrogen and further separated by the normal phase separation method outlined in a previous section.

RESULTS

Normal Phase Separation

A 975 nmol lipid mixture of heptanoamide (FAs), tristearin (TAGs), 1,2-dipalmitoyl-rac-glycerol (DAGs), 1-monopalmitoyl-rac-glycerol (MAGs), N-oleoylglycine (NAGs), palmitamide (PFAMs), and stearoyl ethanolamine (NAEs) single representative standards from each lipid class were separated by normal phase chromatography (**Figure 1**). Sample injection volume was increased from 20 to 200 μL to accommodate larger scale sample purification needs. The effect of the increased injection volume on elution was tested by collecting one fraction per minute over the total gradient elution program. These

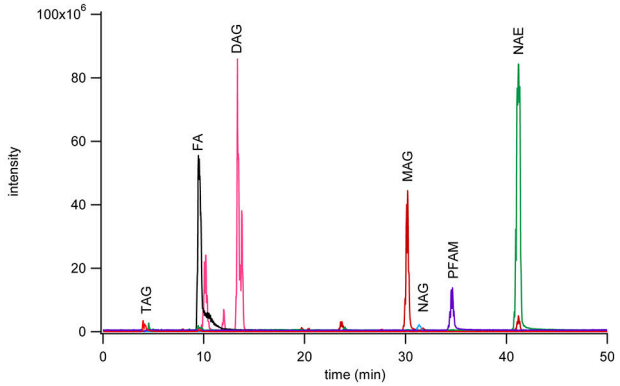

FIGURE 1 | Chromatogram of seven fatty acyl subclasses separated by normal phase chromatography using a YMC PVA-Sil (4.6 × 250 mm, 5 μm particle size) on a Waters ZMD MS with an ESI probe with polarity switching. Separation was achieved with mobile phase A (heptane with 0.5% methyl-tert-butyl ether) and mobile phase B (10% 2-propanol, 0.2% acetic acid in methyl-tert-butyl ether) run in gradient mode from 95 to 50% A over 40 min. The monoacylglycerols were monitored as the $[M+Na]^+$ peak, PFAM as the $[M+H]^+$ peak, diacylglycerols as $[M+Na]^+$ peak, NAE as $[M+Na]^+$ peak, fatty acids as $[M-H]^-$ peak, NAG as $[M-H]^-$ peak, and triacylglycerols as $[M+Na]^+$ peak. Each class was monitored on a different channel on the MS.

fractions were dried down, reconstituted in methanol, and analyzed by reversed phase methods to check the elution range of the desired subclasses. The NAGs and PFAMs between C12 and C22 were found to co-elute from 31 to 38 min. The retention of these lipid classes deviate from that represented in **Figure 1** due to a larger number of individual components in each class, vs. a single representative, and the increase in injection volume causing band broadening. Co-elution was determined not to be problematic because these species ionize in different modes for reversed phase MRM analysis.

Reversed Phase Separation of N-acyl Glycines

Palmitoylglycine (C16:0), oleoylglycine (C18:1^{9}), linoleoylglycine (C18:2^{9,12}), stearidonoyolglyicne (C18:4^{6,9,12,15}), arachidonoylglycine (C20:4^{5,8,11,14}), and arachidoylglycine (C20:0) were separated utilizing a C30 YMC carotenoid column and a fused-core Phenomenex C18 column. Separation of palmitoylglycine (C16:0), oleoylglycine (C18:1^{9}), linoleoylglycine (C18:2^{9,12}), and arachidoylglycine (C20:0) was achieved on a C30 YMC carotenoid column (4.6 × 150 mm, 5 μm particles size) with gradient elution of methanol and water. Both mobile phases were modified with 10 mM ammonium acetate to aid in ionization. Elution was achieved by linear increase in methanol from 90 to 100% over 15 min, followed by a 15 min hold. Elution of each component was determined by identification of the parent mass ion (**Figure 2A**).

In an effort to reduce the analysis time, a fused-core Phenomenex Kinetix C18 column (4.6 × 100 mm, 2.6 μm particles size) was employed using methanol/water gradient elution (**Figure 2B**). The use of a fused-core particle C18 column reduced the experimental time by 6 fold compared to the C30 column separation while simultaneously increasing resolution.

A second elution method was developed (**Figure 2C**) using the fused-core C18 column to determine elution of two additional analytes, arachidonoylglycine (C20:4^{5,8,11,14}) and stearidonoylglycine (C18: 4^{6,9,12,15}). Although the total analysis time increased 4-fold compared with the previous gradient method (**Figure 2B**), co-elution between linoleoylglycine (C18:2) and arachidonoylglycine (C20:4) was reduced.

Utilizing the method developed in **Figure 2C**, the ionization parameters were optimized to yield the lowest detection limit possible. The limit of detection for each analyte was determined by 5 μL injection of standards between 100 nM and 10 μM. The results for limit of detection (LOD = signal to noise ratio, S/N, 5) and limit of quantitation (LOQ = S/N 10) are shown in **Table 1**. The calibration curves consisted of 5 points and were linear ($R^2 \geq 0.994$) between 1 and 10 μM.

Reversed Phase Separation of Primary Fatty Acid Amides

Very long chain PFAMs (C12 to C22) were separated via reversed phase chromatography employing a sub 2 μm particle size column (Agilent RP C18 2.1 × 50 mm, 1.8 μm particle size). A gradient elution was established for a select group

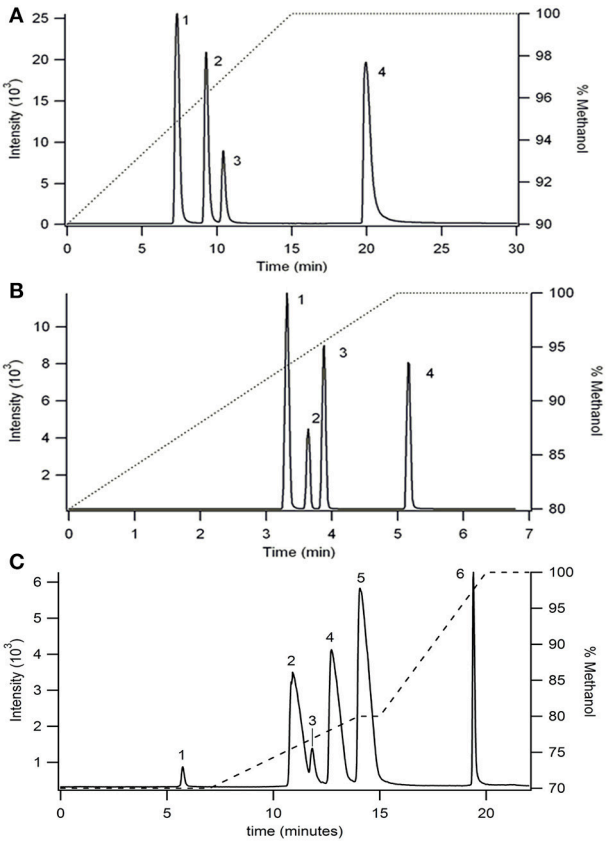

FIGURE 2 | Separation of NAGs on (A) a C30 YMC carotenoid column (4.6 × 150 mm, 5 um particles size) and **(B,C)** Phenomenex C18 column (4.6 × 100 mm, 2.6 μm particles size) using two different gradient separations. Ionized by ESI and detected in MRM mode as the $[M-H]^-$ parent and 74 *m/z* glycine head group product at collision energy of 20 V. Flow rate was 1 mL/min. The right axis shows the gradient elution profile for % methanol. Peak identities in **(A,B)** are (1) linoleoylglycine C18:2, (2) palmitoylglycine C16:0, (3) oleoylglycine C18:1, (4) arichidoylglycine C20:0, and **(C)** are (1) stearidonoylglycine C18:4, (2) linoleoylglycine C18:2, (3) arachidonoylglycine C20:4, (4) palmitoylglycine C16:0, (5) oleoylglycine C18:1, (6) arachidoylglycine C20:0.

of PFAMs (**Figure 3A**), using a methanol/water gradient elution. All components tested were well resolved with the exception of erucamide (C22:1^{13}) and arachidamide (C20:0). Extension of the linear gradient of 80–100% methanol from 5 to 10 min (**Figure 3A**) to 6–20 min (**Figure 3B**) resulted in baseline resolution of erucamide (C22:1^{13}) and arachidamide (C20:0). Due to contamination of samples, as well as blanks and controls, with erucamide, the gradient separation in **Figure 3A** was used for separation of biological samples and erucamide was excluded from the MRM detection method.

Utilizing the separation method developed in **Figure 3A**, the optimized ionization parameters for detection were determined. The LOD for each analyte was determined by 2 μL injection of standards between 500 pM and 10 μM. The results for limit of detection (LOD = S/N, 5) and limit of quantitation (LOQ = S/N

10) are shown in **Table 2**. The calibration curves consisted of 5 points and were linear ($R^2 \geq 0.997$) between 0.500 and 10 μM on average. In cases where the LOD and LOQ were equal, the signal was lost below the LOD, however, at this concentration the S/N was above 10.

Separation and Detection of Primary Fatty Acid Amides in Biological Samples

The developed extraction, normal phase, and reversed phase separation methods were used serially to analyze PFAM content in a sample of mouse brain (**Figure 4**). These studies were conducted to validate the described methodology and ensure the correct method parameters at each of the 3 separate steps, extraction, normal phase lipid class selection, and reversed phase separation and detection. In addition the validation aimed to determine the sensitivity of the method in a complex matrix. Initial attempts failed to reproducibility detect lipids in tissue samples. Several factors in the extraction process were identified which contributed to reproducibility. Plastic materials were avoided due to leachable PFAM contamination (Cooper and Tice, 1995). The samples were frozen and indomethacin was added to the extraction solvent to reduce the rate of PFAM catabolism.

TABLE 1 | LOD and LOQ for several commercially available NAGs determined by injection of 5 μL standard solutions from 100 nM to 10 μM.

N-acyl glycine	Abbreviation	LOD (μM)	LOQ (μM)	R^2
Stearidonoylglycine	C18:$4^{6,9,12,15}$	0.475	1.00	0.999
Linoleoylglycine	C18:$2^{9,12}$	0.499	1.00	0.999
Arachidonoylglycine	C20:$4^{5,8,11,14}$	0.484	1.00	0.999
Palmitoylglycine	C16:0	1.00	1.00	0.994
Oleoylglycine	C18:2^{9}	1.00	1.00	0.999
Arachidoylglycine	C20:0	1.00	1.00	0.999

Calibration curves were linear between 1 and 10 μM. The LOD and LOQ were determined by a S/N of 5 and 10, respectively.

Brain tissue was chosen due to the documented interaction of oleamide and neuroreceptors, such as 5HTR. Myristamide (C14:0), linoleamide (C18:2), palmitamide (C16:0), oleamide (C18:1), stearamide (C18:0), and behenamide (C22:0) were identified in mouse brain tissue extracts by matching retention time and parent ion mass. An internal standard, heptadecanoamide, which was added prior to homogenization of the tissue samples, was recovered at 72% confirming collection of the correct fraction in the normal phase separation. The blank sample had a similar % recovery of the internal standard with no PFAM peaks present (data not shown).

DISCUSSION AND CONCLUSION

This work describes various strategies for efficient separation and detection of saturated and unsaturated fatty acyls. Different subclasses of lipids were resolved with a normal phase separation scheme utilizing a heptane and methyl-tert-butyl ether mobile phase and gradient elution. The addition of isopropanol to the mobile phase was necessary to increase the solubility of these lipids, reducing the carry over between injections. Nonetheless, it was still essential to occasionally wash the column with polar solvents, especially if large injection volumes (>100 μL) were used frequently. The fatty acyl subclasses separated via the normal phase method are those commonly extracted with Folch-Pi from biological samples. This method is comparable to previously reported SPE methods (Sultana and Johnson, 2006) and although it has an increased total analysis time the separation is automated and reproducible. The eluent can be monitored by MS if a post-column feed is used or collected directly for further separation and analyses. It was found that, with a 200 μL injection volume, the PFAMs and NAGs co-elute as a single peak between 31 and 38 min. This, however, was not problematic as subsequent MS/MS studies found that NAGs preferentially ionized in the negative mode while PFAMs were observed in positive mode, allowing easy discrimination between the co-eluting lipids.

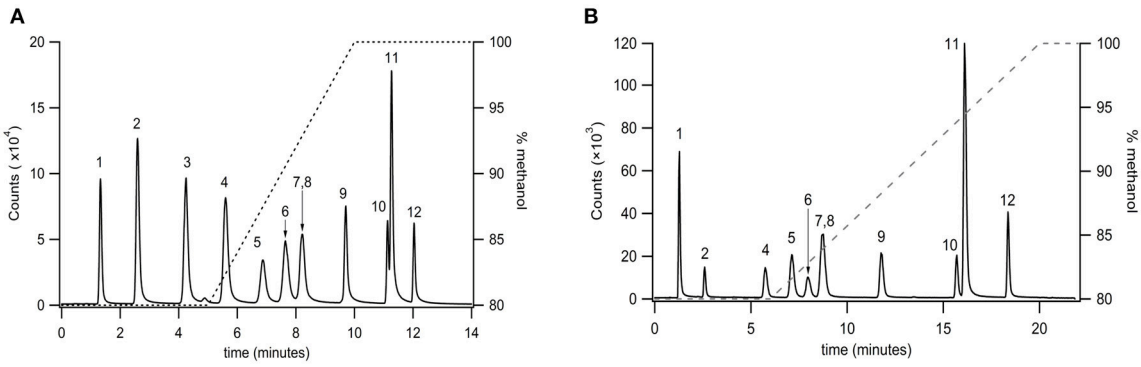

FIGURE 3 | Separation of very long chain PFAMs with Agilent RP C18 column (2.1 × 50 mm, 1.8 μm particle size) using a (A) steep and **(B)** shallow gradient (% methanol on the right axis). Peak identities were identified by MS/MS, with fractions ionized by APCI and identified in MRM mode. The peaks were identified as (1) lauramide, (2) myristamide, (3) linoleamide, (4) palmitamide, (5) oleamide, (6) elaidamide, (7) petroselaidamide, (8) heptadecanoamide, (9) stearamide, (10) erucamide, (11) arachidamide, (12) behenamide.

TABLE 2 | LOD and LOQ for select PFAMs determined by injection of 2 μL standard solutions from 500 pM to 10 μM.

PFAM	Abbreviation	LOD (nM)	LOQ (nM)	R^2
Lauramide	C12:0	50	100	0.999
Myristamide	C14:0	50	50	0.999
Linoleamide	$C18:2^{9,12}$	10	50	0.999
Palmitamide	C16:0	20	50	0.999
Oleamide	$C18:1^{9}$	400	400	0.999
Elaidamide	$C18:1^{9trans}$	40	400	0.998
Petroselaidamide	$C18:1^{6trans}$	40	400	0.999
Stearamide	C18:0	50	50	0.997
Erucamide	$C22:1^{13}$	50	100	0.998
Arachidamide	C20:0	10	50	0.999
behenamide	C22:0	20	20	0.999

Calibration curves were linear between 0.500 and 10 μM. The LOD and LOQ were determined by a S/N of 5 and 10, respectively.

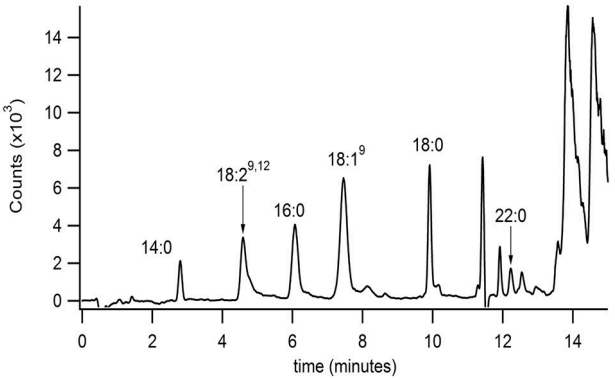

FIGURE 4 | Isolation and identification of PFAMs in brain. The equivalent of 10 Swiss-Webster mouse brains (with mid brain removed) were Folch-Pi extracted. Samples were then subjected to MDLC using normal phase chromatography, and the peak corresponding to PFAM class of lipids were then separated by reversed phase chromatographs (as described in the text). PFAM substituents where identified by MS/MS as described **Figure 3**. PFAM substituents corresponding to 14:0, $19:2^{9,12}$, 16:0, $18:1^{9}$, 18:0, and 22:0 PFAMs were identified in mouse brain tissue by MRM (as described in the text).

Following separation of fatty acyls with normal phase the individual subclasses (e.g., PFAMs or NAGs) can be further separated to determine the distinct analytes present. This was achieved with C18 reversed phase chromatography and detected with tandem MS. The elution order followed the trends observed with fatty acids eluted from reversed phase columns (Gutnikov, 1995). The "critical pairs" were separated by adjusting the gradient elution parameters and/or increasing the column theoretical plates. For PFAMs, isobaric compounds (oleamide $C18:1^{9}$, elaidamide $C18:1^{9trans}$, and petroselaidamide $C18:1^{6trans}$) differing only in double bond position were resolved using a 5 min hold at 80% methanol followed by a 5 min linear ramp to 100% methanol. This resulted in LOD of 10–400 nM was obtained depending on the species. NAGs proved to be difficult to separate on standard C18 columns due to low solubility and increased interaction with the stationary phase. Therefore, a C30 substituted column and a fused-core C18 column with a reduced particle size were employed to determine the optimum conditions for NAG separation. Of note, in our hands NAGs were not found to ionize efficiently or reproducibly in positive ion mode, thus, negative ion mode was used for all NAG studies. The C30 column proved useful in separation of saturated and monounsaturated NAGs with modest tailing, however, the analysis time was undesirable when considering MDLC. The 2.6 μm particle size fused-core column was expected to reduce the analysis time, peak broadening, and the tailing factor. As expected, the analysis time was reduced by 6-fold and peaks were baseline resolved. Due to the low ionization efficiency of these compounds, the LOD was 1 μM for all species, consequently limiting the ability to detect physiological levels.

Utilizing the developed methodology, PFAMs were successfully detected in extracts of mouse brain tissue. PFAMs and NAEs have been reported in biological tissues at pmol/g of tissue. Though the utilized methodology has successfully identified PFAMs in brain tissue, the levels are not sufficient for quantitation. It is clear, even with careful method selection and sample processing consideration, that quantitation of trace levels of the PFAM and NAG subclasses pose a challenge for conventional separation and detection methods. Several factors were found to have a profound impact on analysis. When selectively analyzing for fatty amides the use of all plastic containing equipment and sample vessels should be eliminated. Under Folch-Pi extraction conditions, these substrates leach fatty amides, thus, contaminating the sample matrix (Cooper and Tice, 1995).

Additionally, rigorous care should be taken to fully control experimental conditions before and after tissue excision. Quantitation studies on fatty acid ethanolamines in biological samples have found similar effects with tissue quality (Epps et al., 1979; Skaper et al., 1996; Giuffrida and Piomelli, 1998; Kondo et al., 1998). In our study, addition of indomethacin to the 2:1 chloroform/methanol solvent had a positive effect on detection of components in extracted tissue samples as early extraction attempts without indomethacin did not detect PFAMs in tissue samples. Extra steps were taken to perform extractions when tissues were frozen with the addition of indomethacin to the extraction solution. After these method adjustments, PFAMs were detected in the tissue samples. A cocktail of inhibitors with the use of a trapping MS may provide the sensitivity needed to overcome the LOQ.

In summary, we developed an off-line MDLC system for analysis of PFAMs and NAGs. Using lipid standards, normal phase separation was capable of automated separation of complex biological lipid matrices with comparable sample recovery to SPE. Each individual subclass of lipids could be successfully sampled from the normal phase and further resolved into each individual component with a secondary reversed phase method. Fused core particle and sub-2 μm column packings enabled high resolution separation of lipid lengths from C12 to C22 within 12 min. This included resolution of several positional isomers of C18:1, some of which have been shown to affect physiological

states in vertebrates. The MDLC coupled with MS/MS was then shown to be capable in identifying PFAMs in samples with a more complex lipid composition, homogenized mouse brain. The methodology described herein provides a framework for future analyses aimed at identifying and elucidating the roles and significance of lipids in health and disease.

AUTHOR CONTRIBUTIONS

All experimental studies were conducted by ED, except for the initial normal phase chromatography trials that were conducted by KK. Studies were directed by MC and initial drafts were written by ED and MC.

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Fatty Acid Regulation of Voltage- and Ligand-Gated Ion Channel Function

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Free fatty acids (FFA) are essential components of the cell, where they play a key role in lipid and carbohydrate metabolism, and most particularly in cell membranes, where they are central actors in shaping the physicochemical properties of the lipid bilayer and the cellular adaptation to the environment. FFA are continuously being produced and degraded, and a feedback regulatory function has been attributed to their turnover. The massive increase observed under some pathological conditions, especially in brain, has been interpreted as a protective mechanism possibly operative on ion channels, which in some cases is of stimulatory nature and in other cases inhibitory. Here we discuss the correlation between the structure of FFA and their ability to modulate protein function, evaluating the influence of saturation/unsaturation, number of double bonds, and *cis* vs. *trans* isomerism. We further focus on the mechanisms of FFA modulation operating on voltage-gated and ligand-gated ion channel function, contrasting the still conflicting evidence on direct vs. indirect mechanisms of action.

Keywords: ion channels, cell-surface receptors, ligand-gated channel, fatty acids, PUFA, FFA, VLCFA

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Free fatty acids (FFA) are customarily studied in relation to the physical adaptation of cells to their environment, the normal metabolism of lipids and carbohydrates (lipogenesis, lipolysis, esterification, oxidation, glycolysis, fatty acid uptake) and disease conditions, such as obesity, atherogenic dyslipidemias, type 2 diabetes, or insulin resistance (Boden, 2008). This broad palette is largely associated with the physiology and physiopathology of FFA resulting from the lipolytic breakdown of stored tissular triglycerides. Other diseases involving pathological affectation of fatty acids, albeit rare, include X-linked adrenoleukodystrophy, a progressive neurodegenerative disorder caused by the loss-of-function mutation in the ATP-binding cassette transporter subfamily D member 1 gene. The transporter mediates the transport of saturated very long-chain fatty acids (VLCFA), and the disease displays an abnormal accumulation of VLCFA (Mosser et al., 1993). In addition to these systemic and more general effects of FFA, the activity of many membrane proteins is modulated by the lipid composition of the membranes in which they are embedded and the physicochemical properties of the FFA produced locally are increasingly gaining attention in this regulatory role. FFA are normal constituents of biological membranes that are continuously being produced and catabolized in living cells. No consensus view has emerged to date to account for the mechanisms by which this type of lipids modulate membrane proteins (Ordway et al., 1991; Petrou et al., 1995); it is highly likely that they will turn out to be multifactorial in nature. Under certain stress conditions and most particularly in brain, the amount of endogenous FFA can

Abbreviations: FFA, free fatty acids; nAChR, nicotinic acetylcholine receptor; PUFA, polyunsaturated fatty acids; TM, transmembrane; VLCFA, very long-chain fatty acids.

increase dramatically, a phenomenon that has been interpreted as fulfilling a neuroprotective function (see Lauritzen et al., 2000; Blondeau et al., 2002; Buckler and Honore, 2005). Events like ischemia, hypoxia and kainate-induced epilepsy cause a decrease in the intracellular pH, augmented intracellular FFA concentration and an increase in the cytoplasmic Ca^{2+} concentration, leading to the activation of phospholipases. Lauritzen et al. (2000) showed that the PUFA linolenic acid (n-3 PUFA) prevents neuronal death in an animal model of transient global ischemia even when administered as long as 30 min after the insult. Linolenic acid also protected animals treated with kainate against seizures and hippocampal lesions. The augmentation of both the pH and cytosolic FFA concentrations causes hyperpolarization of the cell membrane and reduces the Ca^{2+} influx, thus deterring excitatory glutamatergic transmission and preventing neuronal death, pointing to the possible role of FFA as neuroprotective agents and anti-epileptic compounds (Lauritzen et al., 2000). In addition, dysfunction in fatty acid metabolism is implicated in disease conditions, such as cardiovascular disease, metabolic syndrome, type 2 diabetes, obesity, hypertension and proimflammatory conditions, as well as in several neurological diseases related to the alteration of insulin equilibrium in brain, as observed in Parkinson's disease, Alzheimer's disease and some forms of the schizophrenic spectrum disorders (Virmani et al., 2015).

It is well documented that FFA can directly or indirectly affect the activity of a variety of ion channels. The former involves the interaction between FFA and the ion channel protein or an associated site within the membrane without intervening factors or intermediaries; indirect effects involve the prior transformation of FFA to biologically active metabolites (Ordway et al., 1991), usually through signaling cascades. FFA effects on ion channels depend on their chemical structure: some ion channels are affected by both saturated and unsaturated fatty acids whereas others are only affected by unsaturated fatty acids (Ordway et al., 1991; Sumida et al., 1993; Meves, 1994). Similarly, *cis* and *trans* FFA isomers have been postulated to exert effects on some ion channels whereas only the *cis* configuration has been identified in other cases (Ordway et al., 1991; Sumida et al., 1993; Meves, 1994). An example of this occurs in the hypertriglyceridemia associated with acute pancreatitis. Hydrolysis of triglycerides by pancreatic lipase in acinar cells releases large quantities of FFA, which trigger an increase in cytosolic Ca^{2+} concentration, an effect that depends on the unsaturated/saturated FFA ratio. High concentrations of unsaturated fatty acids lead to an elevation of cytosolic Ca^{2+} and induce the expression of distinct isoforms of the enzyme phosphokinase C (PKC), the activity of which directly depends on the degree of FFA unsaturation (Chang et al., 2015).

Early fluorescence measurements undertaken by Karnovsky (1979) to study possible membrane alterations induced by FFA led to the classification of FFA into two groups: group A, containing *cis*-unsaturated FFA with a kink in the molecule (such as oleic and palmitoleic acids), and group B, comprising saturated and *trans*-unsaturated fatty acids with a linear structure (such as stearic and elaidic acids). This structural difference has important biophysical implications. Group A fatty acids disorder the membrane's interior and order the more shallow head group region, whereas members of group B do not alter the bilayer core but order the head group region. Such structure-dependent perturbations can lead to conformational changes in membrane-embedded proteins, a fact reflected in various communications reporting differences in biological activity for group A and group B FFA (Casabiell et al., 1991; Pérez et al., 1997, 2003).

Despite the plethora of studies concerning the relationship between FFA and proteins—in particular ionic channels and receptors- it has not been possible to find a common mechanism of action: whereas in some cases FFA stimulate some functional property of the protein in question, in other cases they act as inhibitors. Furthermore, in some cases the structure of the FFA is determinant (saturated vs. unsaturated; one or two vs. more double bonds; *cis* double bonds vs. *trans* double bonds) whereas in other cases the detailed chemical structure does not appear to play a key role; in some cases the FFA acts directly on the target protein whereas in others the FFA does so by activating an intracellular cascade that eventually results in protein modulation. In the following sections, examples of FFA modulation of distinct ion channels will be discussed with special emphasis on the voltage-gated ion channels and the nicotinic acetylcholine receptor (nAChR), paradigm of the rapid ligand-gated ion channels. From the point of view of the *messenger*, in this review we restrict ourselves to the analysis of the modulatory effects caused exclusively by FFA, without dwelling on the changes in the membrane microenvironment produced by the retailoring of the fatty acyl chain composition of either phospholipid or cholesterol ester molecular species. From the point of view of the *target* molecule, we also circumscribe ourselves to the ion channels, leaving aside e.g., the interesting field of G-protein-coupled receptors (GPCRs) acting as sensors of metabolic state, and activated by short-chain fatty acids, i.e., the so-called FFA receptors (Hudson et al., 2013).

FFA EFFECTS ON VOLTAGE-GATED ION CHANNELS

Potassium (K^+) Channels

K^+ channels comprise a large superfamily of integral membrane proteins displaying diverse functions in essentially all organs of vertebrate and invertebrate organisms. Structurally, they possess several transmembrane (TM) helices spanning the lipid bilayer. Based on the combination of their structural and functional characteristics, the K^+ channel superfamily can be divided into three main families: voltage-gated (Kv, 6 TMs), inwardly rectifying (Kir, 2 TMs), and tandem pore domain (K2P, 4 TMs) channels (Kuang et al., 2015). The modulation of these channels by FFA has been documented in numerous instances.

Kv channels are the third-largest class of signal transduction proteins, second only to G protein-coupled receptors (GPCRs) and protein kinases (Yu and Catterall, 2004). The pioneer work of Villarroel and Schwarz (1996) studied the effect of arachidonic acid (AA) over 12 different K^+ channels expressed in *Xenopus laevis* oocytes. Different effects (inhibition or enhancement of the channel current) were observed for each channel; the greatest

effect was observed in the rat K4.2 channel. The current reduction in the presence of AA was near 68%, with an almost total current recovery upon AA removal by washout or bovine serum albumin addition. A similar effect was observed in the presence of 5,8,11,14-eicosatetraynoic acid (ETYA), a nonhydrolizable AA analog. The authors discarded an effect mediated by (1) the metabolic processing of AA by cyclooxygenase, lipooxygenase or epoxygenase pathways, (2) an increment in intracellular Ca^{2+}, or (3) activation of a PKC. By testing different FFA, they also discarded the possibility that the effect of AA on the Kv4.2 channel was mediated by an indirect mechanism related to alteration of the membrane fluidity. The authors postulated a direct mechanism mediated by the specific interaction of AA with the channel; although they could not find the binding site for AA, they suggested that the S4–S5 loop is indirectly involved. In the same work (Villarroel and Schwarz, 1996), the authors described a potentiation effect of AA on the Shaker Kv channel, the prototypic member of the family, with perturbations of the activation and inactivation kinetics, an effect that was not reproduced with ETYA. Later works continue the study of this effect. Modulation by FFA of the Shaker Kv channel shifts the voltage dependence of activation via an electrostatic mechanism that ends in channel activation (Börjesson et al., 2008, 2010; Xu et al., 2008). The observed effect was similar for six different polyunsaturated fatty acids (PUFA), whereas monounsaturated and saturated fatty acids produced no effect (Börjesson et al., 2008). The charge of the PUFA head group determines the direction of the effect, which has been referred to as the "lipoelectric mechanism" (Börjesson et al., 2008, 2010). FFA can also induce channel opening by affecting one or several of the steps/molecular rearrangements leading to channel opening. Studying point-mutated Shaker channels covering the lipophilic surfaces of the extracellular helices of S3, S4, S5, and S6 segments, Börjesson and Elinder (2011) concluded that residues in the voltage sensor domain are important for PUFA acting on the channel's voltage dependence, with high-impact residues clustered in a small region of the lipid-facing S3–S4 bend, where these authors tentatively assign the location of the PUFA site of action. Börjesson and Elinder (2011) further demonstrated that PUFA cause very different effects on different ion channels depending on the presence of charges at specific positions. A recent *in silico* study employing atomistic molecular dynamics simulations identified a similar potential PUFA site in the open channel state of *Shaker* K_V, located at the lipid-facing side of a pocket connecting the extracellular linker between S3 and S4 helices (Yazdi et al., 2016). This study also provided an explanation for the activation caused by FFA: the selective stabilization of the open state of the Kv channel as PUFA established fewer interactions with the protein in the closed state. Interestingly, Yazdi et al. (2016) point out that PUFA induce channel opening by modulating voltage-sensitivity, thus opening a potential therapeutic avenue for the use of ketogenic diets in refractory epilepsy.

Background K^+ channels (4 TMs and 2 pore domain, 2P) play a key role in setting the neuronal membrane potential, in tuning the duration of the action potential and in modulating the membrane input resistance. Kim (2003) reviewed the main characteristics of the modulation of two-pore domain K^+ channels by FFA (TREK-1, TREK-2, and TRAAK). These channels, activated by GPCRs, stretch, pH, and PUFA, are broadly expressed in the nervous system where they control neuronal excitability (Lee et al., 2011; Liu et al., 2014; Blin et al., 2016). The effect of PUFA on TREK channels has profound physiological implications, since TREK-1 was shown to mediate the neuroprotection induced by PUFA (Lauritzen et al., 2000; Heurteaux et al., 2004; Noël et al., 2011; Liu et al., 2014). There are certain structural features that FFA must necessarily satisfy to activate these K^+ channels: the length of the carbonyl group, the unsaturation (saturated FFA are not effective) and the presence of the negatively charged carboxyl group (Patel et al., 2001; Kim, 2003; Noël et al., 2011). It has been demonstrated that the effect of PUFA on fatty acid-sensitive two-pore domain K^+ channels does not require metabolic conversion of fatty acids into other bioactive molecules nor cytosolic messengers, enzymes or Ca^{++} (Horimoto et al., 1997; Maingret et al., 1999; Casavant et al., 2000; Lee et al., 2011). Thus, the effect of FFA could result from direct interaction with the channel protein, via partitioning into the lipid bilayer (e.g., causing changes in membrane fluidity), and/or involving membrane-delimited signal transduction mechanisms (Casavant et al., 2000). Compounds that mimic the effect of AA on membrane properties did not reproduce the effect of FFA on K^+ channels (Patel et al., 2001). TREK and TRAAK are, however, mechano-gated channels that preferentially open by negative mechanical pressure (i.e., convex membrane curvature); it is thus possible that FFA are involved or even induce membrane alterations that lead to changes in membrane curvature (Patel et al., 2001). It has been reported that the cytoplasmic C-terminal region of near 30 amino acids located close to the 4th TM segment of the TREK-1 or TREK-2 K^+ channels confers sensitivity to unsaturated FFA (Patel et al., 1998; Kim, 2003; Lee et al., 2011). Furthermore, using deletion analysis it has been reported that not only the C-terminal group but also the extracellular loop between the 1st TM domain and the 1st pore domain may be important for this effect (Patel et al., 2001).

The large-conductance Ca^{++}-activated K^+ channels (Slo1 BK channels), a very diverse group of channels resulting from alternative splicing in the CNS, are involved in the regulation of multiple physiological processes (Brenner et al., 2000). Similarly, a large number of cellular signaling molecules, including FFA, are known to modulate the function of Slo1 BK channels. For instance, BK channels are activated by FFA in GH_3 cells (Denson et al., 2000), whereas a clonal line derived from GH_3 (GH_4C_1) does not respond to AA, suggesting an alternative BK splice variant in these cells (Duerson et al., 1996). In GH_3, BK channel activation by FFA requires a FA with at least one double bond, and a significant correlation was described between the degree of *cis*-unsaturation and the extent of channel activation (Denson et al., 2000). No significant correlation between changes in membrane fluidity and degree of activation was observed, and no effect of FFA metabolites was detected, leading to the conclusion that *cis*-unsaturated FFA interact directly with the channel protein proper. It was suggested that binding of the FFA to the Ca^{2+} binding site (a domain near the inner surface of the membrane in the cytosolic tail of the

channel) could be responsible for changes of the channel protein conformation and hence changes in Ca^{2+} affinity, resulting in a conformation with a lower energy state (Denson et al., 2000). Further structural features of FFA required to increase BK channel activity have been defined: FFA must have a negatively charged head group and a sufficiently long (C > 8) carbon chain (Clarke et al., 2002). These authors showed that channel modulation is unlikely to be due to an alteration of the membrane electric field or the attraction of local counterions to the channel, concluding that FFA modulation of BK(Ca) channels could occur by direct interaction with either the channel protein or with some other channel-associated component. Sun et al. (2007) postulated a novel mechanism of β-subunit-dependent modulation of BK channels by AA. Further studies also suggested that docosahexanoic acid (DHA) directly activates the Slo1 channel complex with the auxiliary subunit β1 by destabilizing the closed conformation of the ion conduction gate (Hoshi et al., 2013a). Electrophysiological measurements showed that a single residue near the cytoplasmic end of S6 plays a critical role in the DHA activation of human Slo1 as no effect was observed on *Drosophila melanogaster* Slo1 channels, which normally do not contain DHA in the body (Hoshi et al., 2013b). Mutation of Y318, located probably at the cytoplasmic end of S6 in hSlo1 to Y318S, as found in dSlo1, cancels the effect of DHA on hSlo1 (Hoshi et al., 2013b).

One of the initial events in β-cells glucose stimulus-insulin secretion coupling is the closure of the *ATP-sensitive K^+ channel*. It has been demonstrated that an increment in the concentration of FFA results in decreased insulin secretion (Corkey et al., 1989). The effect of FFA was shown to be dual: the initial products of phospholipase A2 digestion, or exogenous FFA, resulted in the reduction of ATP-sensitive K^+ channel activity, whereas i) a long-term activation of these channels by AA, and ii) the reversal of this effect by AA metabolites via the cyclooxygenase pathway are also observed, possibly responding to a counter-regulatory mechanism (Eddlestone, 1995). Overnight incubation of clonal β-cells with palmitic acid induced a 50% decrease in the ability of glucose to stimulate insulin secretion and increased the pool of long-chain CoA (LC-CoA, the metabolically active form of FFA) (Larsson et al., 1996). Oleoyl-CoA increased the mean current 5-fold. Furthermore, the ability of LC-CoA to increase K^+ conductance appears to be specific for the K_{ATP} channel (and not for the big conductance K^+ channel, K_{BK}, which is voltage and Ca^{2+}-dependent, nor for 8-pS K^+ channel). The stimulatory effect of the LC-CoA was dependent on both the acyl group (saturated or unsaturated chains with 14 to 18 carbons) and the CoA component. The accumulation of LC-CoA, possibly as a consequence of high glucose exposure (which inhibits fatty acid oxidation and elevates cytosolic LC-CoA levels), could be causally related to a loss of responsiveness to glucose. In tissue culture or non-insulin-dependent diabetes mellitus, long-term exposure to FFA impairs glucose-induced insulin by preventing the closure or promoting the opening of K_{ATP} channels (Larsson et al., 1996). LC-CoA esters with a chain length exceeding 12 carbons were also observed to be potent activators of the K_{ATP} channel in human pancreatic beta cells (Bränström et al., 2004). Previously it was demonstrated that fatty acid activation of ATP-sensitive K^+ channels was most likely due to the participation of AA (and other *cis*-unsaturated fatty acid)-activated protein kinase C (PKC) isoenzymes and not by metabolites of AA via the cyclooxygenase or the lipoxygenase pathways (Müller et al., 1992). Similarly, a direct connection between AA and PKC activation was experimentally tested, explaining the mitogenic potential of AA (4-fold increase in DNA synthesis) in rat brown preadipocytes (Garcia et al., 2012). AA was in fact shown to activate PKC in various tissues, albeit by different mechanisms of action (Sekiguchi et al., 1987; Shinomura et al., 1991; Chen and Murakami, 1992; Blobe et al., 1995; Nishizuka, 1995; Nowicki et al., 1997; Leu et al., 2010). AA inhibition of Na^+-K^+-ATPase in sheep pulmonary artery was postulated to be mediated mainly by 20-HETE, the major metabolite of cytochrome P-450-arachidonic acid ω-hydroxylase pathway, through the activation of PKC (Singh et al., 2012). A similar mechanism was postulated in rat proximal convoluted tubules (Li et al., 2000). Nowicki et al. (1997) postulated that this inhibition was mediated by PKC-phosphorylation of Ser23 on the Na^+, K^+-ATPase α subunit. Another study suggested that 20-HETE can lead to PKC-dependent phosphorylation of Ser23 in Na^+-K^+-ATPase and of Ser896 in NMDA receptor NR1subunits at the putamen of piglets (Yang et al., 2012). A further study demonstrated that AA improved prostate cancer cell survival through 5-lipoxygenase (5-LOX) metabolites, a process which involved downstream PKCε activity (Sarveswaran et al., 2011). These authors further showed that treatment of prostate cancer cells with MK591, a 5-LOX inhibitor, or 5-LOX shRNA not only decreased PKCε expression but also diminished membrane localization of PKCε, inducing apoptosis. PKCε may thus be a mediator of survival signals downstream of 5-LOX metabolites (Sarveswaran et al., 2011).

Fatty acids act as substrates for acyl-coenzyme A (acyl-CoA) molecules by several synthases. These activated fatty acids can be short-chain (acetyl-CoA), medium-chain (e.g., octanoyl-CoA) or long-chain acyl-CoAs, such as palmitoyl-CoA. The effect of FFA and acyl-CoA esters on a highly active *plant* mitochondrial *ATP-sensitive K^+ channel* (PmitoKATP) was studied in mitochondria isolated from durum wheat (Triticum durum Desf.) (Laus et al., 2011). Acyl-CoAs, linoleate and other FFA (laurate, palmitate, stearate, palmitoleate, oleate, arachidonate, and the non-physiological 1-undecanesulphonate and 5-phenylvalerate) directly activate PmitoKATP, but not through the Plant Uncoupling Protein (PUCP). The same activation effect was found to be widespread in mitochondria from different plant species and organs. Since PmitoKATP may act against environmental/oxidative stress (Atkin and Macherel, 2009), FFA activation in plants has been proposed to represent a physiological anti-stress mechanism: under hyperosmotic (NaCl or mannitol) stress conditions, FFA increase and may activate PmitoKATP strongly (Laus et al., 2011).

In rat atrial myocytes, perfusion of the cytoplasmic face of the membrane with unsaturated FFA (10–50 μM) such as AA, linoleic, and eicosatrienoic acids was reported to inhibit the ATP-sensitive K^+ channel almost completely; lysophospholipids also markedly inhibited channel openings. In contrast, AA activated the *ATP-insensitive K^+ channel* with an outwardly rectifying

property. Since the FFA levels rise after long periods of ischemia, the authors speculated that the ATP-insensitive K^+ channels contribute to a late phase of extracellular K^+ accumulation (Kim and Duff, 1990).

Ca^{++}-dependent basolateral membrane K^+ channel (K_{Ca}) activation in intestinal cells induces membrane hyperpolarization and a Cl^- secretory current. It was demonstrated that AA is a second messenger in this pathway: AA levels increase via Ca^{2+}-dependent agonists through different pathways (PLA2, DAG, or DAG lipase) and induce a temporal modulation of Cl^- secretion. The inhibition of K_{Ca} can occur both through the extracellular or intracellular side of the channel and does not depend on the generation of either cyclooxygenase or lipooxygenase metabolites (Devor and Frizzell, 1998). This K_{Ca} inhibition was not specific for AA, as other FFA showed similar effects albeit to a lesser extent. A FFA effect caused by membrane fluidity changes was discarded as a plausible hypothesis, because different FFA act in a similar way on K_{Ca} but induce deviations from the optimal membrane fluidity in either direction (Devor and Frizzell, 1998). Hamilton et al. (2003) concluded that AA exerts a direct effect on K_{Ca} channel (identified as hIK1/hSK4) and described a second-messenger binding site for AA and other FFA. The AA sensitivity of hIK1 lies within the S5 pore and the S6 region, two amino acids (Thr^{250} and Val^{275}) being crucial for this modulation. The side chains of both amino acids extend into the hIK1pore, suggesting that AA causes a direct pore block.

A medically important area of FFA modulation of ion channels is without doubt the mechanism underlying the anti- or pro-arrhythmic effects exerted on cardiac ion channels. PUFA were shown to reduce membrane electrical excitability of neonatal cardiac myocytes and provide an electrophysiological basis for the antiarrhythmic effects of these fatty acids (Kang et al., 1995). Research in this field has developed rapidly and insights into the action of FFA on cardiac myocytes have exploded. An example is the multiple and complex series of effects observed after *n*-3 supplementation, summarized by Moreno et al. (2012): "*n*-3 PUFA inhibit the fast sodium current (I_{Na}), ultrafast activating delayed outward potassium current (I_{Kur}), transient outward potassium current (I_{to}), rapidly activating delayed rectifying outward potassium current (I_{Kr}), L-type calcium inward current (I_{Ca}), and NaC-Ca2C exchange current (I_{NCX}), and enhanced slowly activating delayed rectifying outward potassium current (I_{Ks}) and inward rectifying potassium current (I_{K1})." It is still not clear, however, which is the mechanism operative in n-3 FFA modulation of ion channels: is it a direct or an indirect effect? The prevalent opinion is to consider a direct interaction of FFA with the ion channel protein proper (Moreno et al., 2012 and references therein). Guizy et al. (2005) reported a blocking effect of both AA and DHA on human *ether-a-go-go*-related gene (HERG) channels, whose activation determines the duration of the action potential. This effect, which was found to depend on time, voltage and channel conformational state, was compatible with an open-channel block mechanism. The fact that ETYA caused a similar blockage effect suggests that these PUFA act through a direct mechanism and not through AA metabolism. Clearly, this mechanism requires the channel to be in an open state, but changes in the channel gating parameters suggest that these FFA also interact with the closed state. The inhibition action of these FFA on HERG channels can help to further explain the reported antiarrhythmic effects of AA and/or DHA (Guizy et al., 2005). Open-channel blockage by polyunsaturated FFA, apparently by binding of the FFA to an external site in the channel, was also reported for the major *voltage-dependent K^+ channel (Kv1.5)* cloned from cardiac cells (Honoré et al., 1994; Guizy et al., 2008). In contrast to this effect, Gavrilova-Ruch et al. (2007) reported that AA activated human *ether à go-go* (hEAG) potassium channels expressed in CHO cells, an effect totally reversed upon washing with BSA. The potentiation effect was directly dependent on the number of *cis*-double bonds in the FFA, and probably involved a direct mechanism of action, as ETYA also potentiated hEAG currents, presumably acting through the outer membrane leaflet. The fact that FFA activate hEAG channels whereas they inactivate HERG channels may be due to structural differences in the pore mouth which, in the case of hEAG channels, would prevent the access of AA to the permeation pathway. These channels are normally expressed in neuronal tissue but also in various tumoral tissues, pointing to a possible oncogenic role; the activating effect of AA on EAG channels has therefore been related to an enhanced tumor proliferative rate (Gavrilova-Ruch et al., 2007).

Anion Channels

Anion channels, *ClC-2 Cl^- channels* in particular, are widely distributed in epithelial and non-epithelial tissues. One of the potentially lethal, inherited diseases affecting Cl^- channels is cystic fibrosis. The disease is caused by mutations in the gene encoding a cAMP-regulated, phosphorylation-gated Cl^- channel, the *cystic fibrosis transmembrane conductance regulator (CFTR)*. CFTR-mediated Cl^- and bicarbonate transport drives fluid secretion across epithelial cells. In the gastrointestinal tract, the pathological alteration or loss of CFTR function severely hampers the production of exocrine pancreatic and intestinal secretions, leading to incomplete food digestion and malabsorption. In CF newborns, the defective fluid secretion may lead to a life-threatening obstruction of the distal small intestine, the so-called meconium ileus syndrome. In the pulmonary tract, the CFTR plays a major role in maintaining the airway surface protected by a fluid biofilm. It is therefore not surprising that the respiratory tract is often the target of secondary infections and pneumonia by the combination of CFTR and defective mucosal immunity, *Pseudomona aeruginosa* being the main pathogen involved. The CFTR is inhibited by several fatty acids in the following order: linoleic $\geq$ arachidonic $\geq$ oleic $>$ elaidic $\geq$ palmitic $\geq$ myristic (Linsdell, 2000). The mechanism of AA inhibition has been suggested to result from the electrostatic interaction of the FFA with positively charged amino acids located at the cytoplasmic vestibule of the CFTR channel pore (Zhou and Linsdell, 2007), pointing to a more general inhibitory mechanism of apical membrane Cl^- channels by different FFA, which would act from the cytosolic surface thus rejecting the idea that inhibition results from changes in membrane fluidity (Anderson and Welsh, 1990).

Mutations in the CFTR Cl^- channel are associated with severe lung disease in about 5% of cystic fibrosis patients, a condition that may lead to death. Consequently, a possible therapeutic approach for cystic fibrosis is the potentiation of alternative pathways for Cl^- transport in the lung, including the targeting of ClC-2 Cl^- channels in the epithelium of the respiratory tract. These channels were found to be activated by oleic, elaidic and arachidonic acids and by cAMP-dependent PKA, but AA was found to increase the Cl^- currents in a PKC-and PKA-independent manner (Tewari et al., 2000). The increment of Cl^- currents was FFA dose-dependent, and only observed with unsaturated FFA indistinctly of *cis* or *trans* isomerism. The authors postulated that the effect of FFA may be due to direct effects on the channels, probably through mechanisms similar to those underlying the effect of FFA on the TREK-1 channel described above, and not to products of FFA metabolism (Tewari et al., 2000).

Sodium (Na^+) Channels

Cardiac Na^+ channels (the major class of ion channels that determines cardiac excitability) are also modulated by FFA, causing a reduction in the electrical excitability and/or automaticity of cardiac myocytes. Here again, it is postulated that the inhibition is dependent on FFA structure (Kang et al., 1995; Xiao et al., 1995), reversible in the presence of BSA, and not mediated by FFA metabolites (Kang and Leaf, 1996; Xiao et al., 1997). The mechanisms by which they exert their action remain uncertain; however, it is postulated that FFA act as non-competitive inhibitors, through a single class of sites, by an allosteric inhibitory mechanism (Kang and Leaf, 1996). These authors postulated a model in which the FFA hydrophobic portion interacts with the hydrophobic TM domains of the channel protein at either the lipid-channel interface or at the space between hydrophobic protein domains; the negatively charged carboxyl group interacts ionically with the positively charged amino acid residues of the channel protein near the surface of the bilayer where the carboxyl group is anchored. Cardiac Na^+ channels consist of two subunits: the α subunit (the largest one, itself constituting a functional channel) and the β subunit (smaller; it interacts functionally with the regulatory segments of the Na^+ channel) (An et al., 1998). FFA rapidly and strongly suppress voltage-gated Na^+ currents in cells transfected with only the α-subunit of the human cardiac Na^+ channel and prolong the duration of its inactive state, probably by binding to the inactivated form of the channel (in this state the channel displayed a 43-fold higher affinity for FFA than channels in the resting state) (Xiao et al., 1998). However, not only PUFA but all FFA exerted effects, leading these authors to the conclusion that the characteristic specificity of the effects of PUFA on native Na^+ currents was lost in the exclusive presence of the Na^+ channel α subunit. Xiao et al. (1998) also postulated that the configuration of the α-subunit may be more open or uncovered in the absence of other components of the intact voltage-dependent human cardiac Na^+ channel, thus allowing even those FFA lacking the two or more double bonds to gain access to the site(s) at which PUFA affect conductance in the complete channel. The authors suggested that a short cytoplasmic segment of the transfected channel, linking the III and IV TM segments, was the site of action of FFA (Xiao et al., 1998). Co-expression of the β-subunit with the α-subunit of the human cardiac Na^+ channel restores the selective effect of the PUFA, leading to the conclusion that the β-subunit modifies the FFA blockage of the Na^+ channel (Xiao et al., 2000). A study with the hH1(α) Na^+ channel led to the discovery of the importance of Asn 406 in the inhibition of cardiac voltage-gated Na^+ currents by PUFA (Xiao et al., 2001). Another study using cells transfected with the *skeletal muscle sodium channels* (SkM1) isoform showed that ion channel modulation by FFA depends on the mode of FFA administration (Wieland et al., 1996). Intracellular AA exposure increased channel currents whereas the hH1 isoform did not show significant current increases. Thus, the response to FFA must include an isoform-specific element. In contrast, both isoforms were inhibited when unsaturated FFA were applied extracellularly. These results point to the existence of two distinct sites and mechanisms for FFA modulation of sodium channels: a potential extracellular site for extracellular FFA, and an intracellular one that appears to be exclusively for SkM1. The activation effect was observed over 120 min, suggesting that neo-synthesis and trafficking/insertion of new channels to/in the cell membrane could occur during this period, alone or in combination with unmasking of reserve channels already present in the plasmalemma (Wieland et al., 1996). This dual mechanism was also observed in the muscle *rNa(V)1.4* channel isoform, which appears to depend on the depolarizing potential: AA, but not its metabolites, increased the channel current evoked by a −30 or −40 mV depolarization of the membrane potential, but significantly decreased it by a depolarization over −10 mV (Gu et al., 2009). It was also reported that *cis*-unsaturated FFA with a double bond at position 9 have a biphasic effect on connexin 46 hemichannels: current activation at low FFA concentration and current inhibition at higher concentrations, the effect being directly proportional to the number of double bonds (Retamar et al., 2011). The authors ruled out the possibility that the biphasic effect was mediated by changes in the biophysical properties of the plasma membrane. They concluded that activation and inhibition current mechanisms involve different sites of action (Retamar et al., 2011).

A different dual mechanism of AA action has been postulated for the modulation of δ-opioid receptor (DOR) function (Sullivan et al., 2015). This modulation involves regulation by cyclooxygenase (COX) and lipoxygenase (LOX) dependent metabolites and activation of PKC. A COX-dependent metabolite of AA induces a responsive state of DOR that is capable of mediating antinociception and inhibition of adenylyl cyclase activity in response to opioid agonists, whereas a novel LOX-dependent metabolite of AA produces a loss of responsiveness of the DOR system. Sullivan et al. (2015) further demonstrated that exogenously added and endogenously produced AAs follow different metabolic processes, pointing to the existence of subcellular compartmentation of the enzymes involved. This dual regulation of DOR function may explain the observed variations in the efficacy of opioids in the treatment of pain. Another dual mechanism of FFA is described below for nicotinic acetylcholine receptors.

FFA EFFECTS ON IONOTROPIC NEUROTRANSMITTER LIGAND-GATED ION CHANNELS

The regulation of rapid ligand-gated ion channels by fatty acids is illustrated using two paradigmatic cases, the γ-amino butyric acid receptor and the nicotinic acetylcholine receptor.

γ-Amino Butyric Acid Receptor (GABA-R)

Exogenously added unsaturated FFA, or endogenously produced by the cell, modulate the GABA-R by drastically altering the binding characteristics of various GABA-R ligands, underlying the importance of the lipid environment for this process (Schwartz et al., 1988; Koenig and Martin, 1992; Samochocki and Strosznajder, 1993; Witt and Nielsen, 1994; Witt et al., 1996). FFA acting on the GABA-R must have at least one C-C double bond and a carbon length of 16–22 C (Witt and Nielsen, 1994). The effect of FFA on GABA/benzodiazepine receptor Cl^- channel complex from mammalian, avian, amphibian, and fish species was studied *in vitro* (Witt and Nielsen, 1994). Different effects of unsaturated FFA were observed on [^{3}H]diazepam and [^{3}H]muscimol binding: FFA enhanced ligand binding in the case of mammalian and amphibian receptors; of 17 fish species studied, 11 species presented weak stimulation of ligand binding, 4 species did not show augmented stimulation and 2 species exhibited inhibition; in the 10 bird species studied, only weak enhancement of [^{3}H]muscimol binding was found, whereas [^{3}H]diazepam binding was similar to mammal species (Witt and Nielsen, 1994). These results point to phylogenetic differences in the receptor that might account for the differences in FFA modulation. Again it is possible to consider a direct or an indirect FFA mechanism for GABA-R modulation. The argument for indirect effects rests on the fact that ontogenetic differences also involve changes in membrane composition, as fish membranes are composed mainly of phospholipids with unsaturated fatty acids and, hence, a further fluidizing effect by exogenously added unsaturated FFA is unlikely (Witt and Nielsen, 1994). However, an increase in the temperature of the binding assay, which induced an increment in membrane fluidity, did not alter the unsaturated FFA effect on ligand binding to GABA-R. Evidence pointing to a direct mechanism included the study of FFA effects on distinct recombinant human $GABA_A$-R complexes formed by different subunit compositions (α, β, and γ subunits) where a modulation of the ligand binding by FFA was dependent on the subunit combination. These results suggest the existence of specific amino acid sequences in the α subunits that confer FFA sensitivity to the $GABA_A$-R (Witt et al., 1996, 1999). Additional data suggest that FFA bind to specific sites, unleashing apparently independent responses: the rapid potentiation of the GABA currents and the increased desensitization of the GABA-R complex, which requires the presence of the γ2 subunit (Nabekura et al., 1998). Thus, the combination of different factors, such as the type and concentration of FFA plus the various combinations of GABA-R subunits in the same neuron result in a broad spectrum of potential modulatory mechanisms which might affect GABA responses in the CNS. The mechanism of FFA modulation on $GABA_A$ receptors –activation of second messenger systems- appears to be shared by another inhibitory ligand-gated ion channel, the glycine receptor (Kloda et al., 2007).

Nicotinic Acetylcholine Receptors (nAChRs)

"A protein isolated from Naja naja siamensis venom on the basis of its phospholipase A activity inhibits acetylcholine receptor function in post-synaptic membrane vesicles from *Torpedo californica*" (Andreasen and McNamee, 1977). This was the first evidence linking FFA with nAChR function. Shortly after, it was demonstrated that incorporation of unsaturated fatty acids or lyso-phosphatidylcholine into *Torpedo* membranes also inhibited nAChR function whereas lyso-phosphatidylethanolamine and most saturated FFA caused no effect (Andreasen et al., 1979). This inhibitory effect could be reversed and/or prevented by treatment with bovine serum albumin. Spin-labeled fatty acids also inhibited *Torpedo* nAChR, the magnitude of this effect being largely dependent on the position of the nitroxide group along the hydrocarbon chain (Andreasen and McNamee, 1980). The FFA inhibitory effect on nAChR function was attributed to the perturbation of protein-lipid interactions, and the magnitude of the effect was found to depend on FFA structure (Andreasen and McNamee, 1980). Alterations in nAChR function by FFA, particularly linolenic acid, or by phospholipase A2 hydrolysis products were directly correlated with perturbations of nAChR structure (Villar et al., 1988).

We analyzed the effect of four long-chain free fatty acids (AA, 20:4; DHA, 22:6; palmitic acid, 16:0; and nonadecanoic acid, 19:0) on the function of the acetylcholine receptor (nAChR) at the single-channel level (Bouzat and Barrantes, 1993a). The effect had a rapid onset and only very brief opening events were apparent after FFA application. The modification appeared not to be critically dependent on the degree of FFA saturation. In intact cells, fatty acids could reach and affect nAChR channels in the plasmalemma under the patch pipette when added from outside the patch-clamped area, suggesting i) that fatty acids diffused laterally and ii) the possibility that the AChR-lipid interface was the site of action of FFA (Bouzat and Barrantes, 1993a).

Free fatty acids (FFA) display the highest affinity for the native membrane-bound nAChR among all lipids studied to date (Marsh and Barrantes, 1978; Ellena et al., 1983; Dreger et al., 1997; Mantipragada et al., 2003). We subsequently disclosed the occurrence of independent sites for phospholipids and sterols in native nAChR membranes (Antollini and Barrantes, 1998), and found that these discrete sites were both accessible to FFA. From fluorescence quenching studies using nitroxide spin labels we also tentatively concluded that the sites were located at a shallow depth close to the phospholipid polar head region in native nAChR membranes (Barrantes et al., 2000). However, despite being located at the same site, each class of FFA differs in its effect on the physical properties of the membrane depending on its structure. Using the polarity-sensitive fluorescence probe Laurdan, it was possible to distinguish between saturated FFA,

which induced a small increase in membrane order, and *cis*-unsaturated fatty acids, which caused a clear decrease of the lipid order. Double-bond isomerism could also be distinguished: oleic acid (18:1*cis*) induced a net disordering effect, whereas elaidic acid (18:1*trans*) produced no changes in membrane order (Antollini and Barrantes, 2002). These data lead us to suggest that it is the direct action of FFA at the lipid-protein interface, displacing essential lipids from their sites, rather than changes in bulk properties, such as membrane fluidity, that accounts for the inhibitory effect of FFA on nAChR function.

Two types of lipid sites have been described to be present in integral membrane proteins in general, and at the lipid-nAChR interface in particular: annular and non-annular sites. Annular sites constitute the first shell of lipids surrounding the protein and interact with the protein in a relatively less specific manner; the rate of exchange between annular shell lipid and bulk membrane lipid is relatively fast, in the order of $1–5 \times 10^8$ s^{-1} (Marsh and Barrantes, 1978; Barrantes, 2004). Non-annular lipid sites involve spaces between TM helices and between subunits in multisubunit proteins (Lee, 2004). Non-annular lipids are considered essential for protein activity, and display higher specificity for the protein. The rate of exchange of non-annular lipids with bulk lipids has not yet been experimentally determined, but is presumably sluggish, and in any case much slower than that of annular lipids, as a result of the high specificity of the interaction between non-annular lipids and the protein (Lee, 2004). On the basis of competition studies, early studies suggested that non-annular lipids were associated with binding sites to which cholesterol is bound but phospholipids are not (Jones and McNamee, 1988). We demonstrated that both endogenous FFA generated by phospholipase A2 from *Torpedo* native membranes and AA exogenously added to these membranes localize at both annular and non-annular sites at the lipid-protein interface (Fernández Nievas et al., 2007). Furthermore, we found that nAChR conformational transitions between the resting (R) state and the desensitized (D) state may entail a rearrangement of the nAChR TM region involving the occlusion of non-annular sites at the lipid-protein interface or simply decreased lipid efficacy in accessing such sites (Fernández Nievas et al., 2007; **Figure 1**).

Taking advantage of the different affinities that the fluorescence dye crystal violet (CrV) displays for the resting (R) and desensitized (D) states of the nAChR we observed that the dissimilar effects that FFA exert on the receptor conformational states depend on the structural characteristics of the fatty acids (Fernández Nievas et al., 2008). Whereas *cis*-FFA increased membrane polarity, *trans*-FFA and saturated FFA caused essentially no changes in this property. Only *cis*-FFA drove the nAChR out of the R state in the absence of agonist; we hypothesized that most likely direct contacts between the FFA and TM portions of the nAChR are responsible for driving the receptor out of the R state and, hence, inhibit its function. *cis*-FFA caused a second effect on membrane-bound nAChRs: they prevented the receptors from reaching the D state in the presence of agonist. This lack of transition to the D state of the nAChR could be mimicked by raising the temperature, which disorders the membrane bilayer, or by treatment with PLA2, which decreases the polarity of the membrane, two conditions that do not perturb the R state of the nAChR. The second effect seems not to depend on the presence of specific molecules at the lipid-nAChR interface but rather to be unspecific, mainly associated with changes in the physical state of the bulk membrane. Thus, whereas foreign molecules at the lipid-protein interface probably modify the "activation gate" of the nAChR-associated channel, leading to an intermediate D state, changes in the physical state of the membrane (particularly changes in membrane order or polarity) are likely to perturb the "desensitization gate" (Fernández Nievas et al., 2008; **Figure 2**). A previous study with single cells obtained from flexor digitorum brevis muscles of adult male mice also correlated nAChR functional inhibition with nAChR protein conformation (Nojima et al., 2000). These authors demonstrated that AA, and prostaglandin D2 (PGD2) or its metabolites, cooperatively accelerate desensitization of the nAChR. The explanation provided invoked the activation of PKC by AA and PGD2 in this mechanism. However, the experiments of Fernández Nievas et al. (2008), performed in isolated nAChR-rich plasma membranes in the absence of phosphorylation (absence of ATP and insufficient Mg^{2+} concentration; Safran et al., 1990) clearly demonstrated the inhibition and conformational changes of the nAChR, suggesting that the inhibition and desensitization of the nAChR by FFA does not involve PKC activity. Putting all the information together reinforces the view that there is more than one mechanism of action involved in the modulation of nAChR function by FFA.

In order to further dissect the mechanisms of action of *cis*-unsaturated FFA on the native membrane-bound *Torpedo* nAChR, we resorted to the use of five different monounsaturated fatty acids with the same number of carbon atoms (*cis*-6-18:1, *cis*-9-18:1, *cis*-11-18:1, *cis*-13-18:1, and *trans*-9-18:1) (Perillo et al., 2012). Four out of five 18:1 FFA tested (*cis*-9-18:1, *cis*-11-18:1, *cis*-13-18:1, and *trans*-9-18:1) were found to localize at both annular and non-annular sites; only *cis*-6-18:1 was found in annular sites. Membrane order was found to undergo a very slight and saturable decrease in the presence of the *trans*-unsaturated FFA, whereas all *cis*-monounsaturated FFA tested caused membrane order perturbations dependent on the position of the double bond. The largest effect was observed when the double bond was near the middle of the acyl chain. Patch-clamp experiments disclosed the inhibition of nAChR currents only with *cis*-9-18:1 or *cis*-6-18:1. All unsaturated FFA prevented the nAChR from reaching the D state in the presence of agonist but only *cis*-9-18:1, *cis*-11-18:1, and *cis*-13-18:1 drove the AChR out of the R state in the absence of agonist. Finally, only *cis*-monounsaturated FFA caused a local topological change in the nAChR γTM4 segment (*cis*-6-18:1 to a lesser extent) (**Table 1**). Taken together, these data led us to conclude that the position of the torsion angle of unsaturated FFA is a key factor in channel blockage. If one takes into consideration that (i) the sequence of structural events coupling ligand binding to channel gating begins with movements at the ligand-binding loops, is followed by the displacement of loops located at the interface between the extracellular ligand binding domain and the TM domain, the

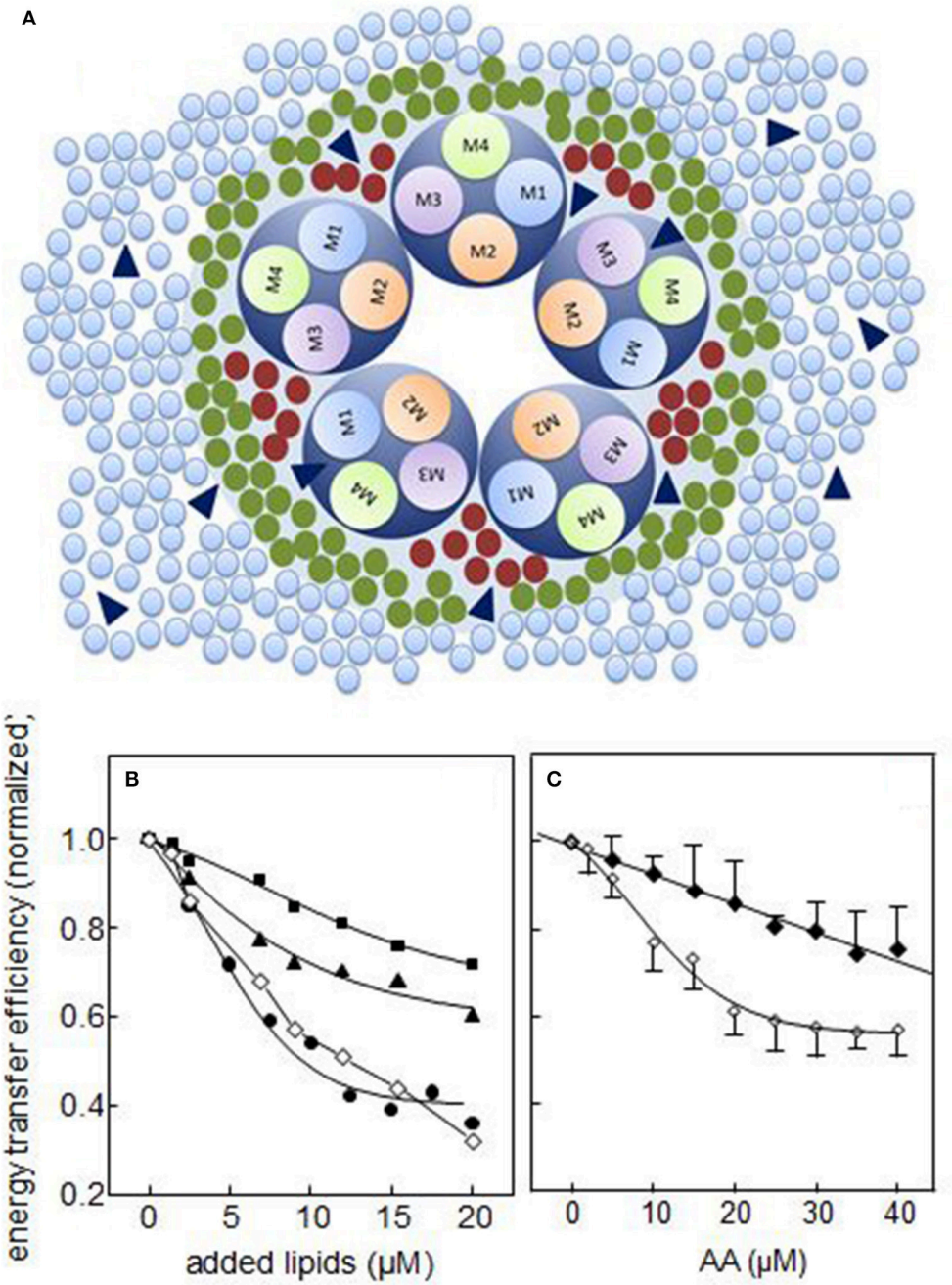


FIGURE 1 | (A) Schematic diagram of a direct mechanism of action of FFA on nAChR function. The scheme illustrates the nAChR-lipid relationship in a receptor-rich membrane, outlining the spatial distribution of the transmembrane segments (TM1, TM2, TM3, and TM4) and the surrounding lipid shell. Three lipid topologies are indicated: non-annular lipids (bordeaux •), annular lipids (green •) and bulk lipids (light blue •). FFA (blue ▲) can be found in any of these three domains. **(B,C)** Experimental studies on annular and non-annular sites using the efficiency (*E*) of the Förster resonance energy transfer (FRET) process between the intrinsic fluorescence of *T. californica* nAChR membranes and the extrinsic fluorescent probe Laurdan; **(B)** in the presence of increasing concentrations of DOPC (■), cholesterol hemisuccinate (CHS, ▲), and oleic acid (•), where the symbol (◊) corresponds to the sum of *E* of DOPC and CHS (from Antollini and Barrantes, 1998) and **(C)** in the presence of increasing concentrations of arachidonic acid with the nAChR in the resting ("R", ◊) or the desensitized ("D", ♦) state. The latter was generated by incubation of the membrane with 1 mM carbamoylcholine prior to the fluorescence measurements (From Fernández Nievas et al., 2007).

subsequent tilting/bending of the pore-lining M2 helix, and ends with movements of M4, M3, and M1 helices in the TM domain (Mitra et al., 2004; Auerbach, 2005), and (ii) that FFA might exert their action by an allosteric mechanism at the lipid-nAChR protein interface, those FFA with the double bond at a shallow position in the membrane probably share the topological loci of

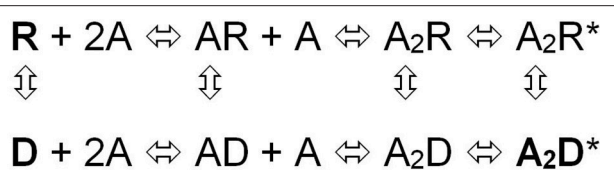


FIGURE 2 | Sequential model of the nAChR activation equilibrium. The model considers that each state of channel activation has a corresponding desensitized state (Dilger and Liu, 1992): R is the nAChR in the resting state, A the agonist and RA and RA2 represent the nAChR with one or two agonist molecules bound, respectively, and RA2* is the biliganded nAChR in a transient active open configuration; D, AD, A2D, and A2D* are the corresponding isoforms in the desensitized, non-conductive states, respectively. This allosteric equilibrium can be affected e.g., by single-point mutations and exposure to some drugs (From Fernández Nievas et al., 2008).

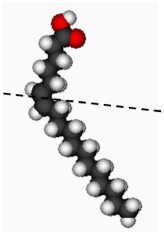
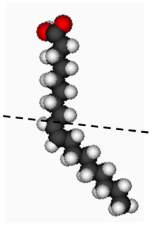
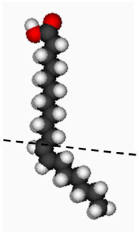
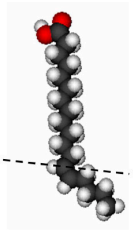
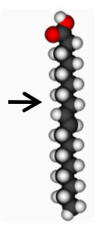
the conserved core structure for nAChR gating and hence could perturb this synchronous mechanism (Perillo et al., 2012).

Changes in membrane lipid composition (Criado et al., 1984; Sunshine and McNamee, 1994; Baenziger et al., 2000; da Costa et al., 2002) and/or the presence of exogenous hydrophobic molecules like steroids and fatty acids (Andreasen and McNamee, 1980; Villar et al., 1988; Bouzat and Barrantes, 1993a,b, 1996; Lasalde et al., 1995; Nurowska and Ruzzier, 1996; Santiago et al., 2001; Garbus et al., 2001, 2002) affect AChR function. Individual amino acid mutations in the lipid-facing outer ring (M4) of the nAChR TM domain also modulate nAChR function (Lee et al., 1994; Lasalde et al., 1996; Ortiz-Miranda et al., 1997; Bouzat et al., 1998; Tamamizu et al., 1999, 2000), a clear indication that although far away from the agonist sites and from the ion channel, the M4 TM segment, the only member of the outermost TM ring (Barrantes, 2003), effectively influences nAChR function. In this sense, M4 would behave as a sensor of the lipid-protein environment unleashing a signal to the M2 channel region, probably initiated by the induction of changes in the topology of the outer ring and ultimately causing a conformational change of the whole AChR (Xu et al., 2005; Fernández Nievas et al., 2007, 2008; Perillo et al., 2012; Barrantes, 2015).

Contrary to the rapid inhibitory effect described above, a potentiation effect of AA on *Torpedo* nAChR expressed in *Xenopus* oocytes, lasting over 30 min after FFA washing, has been reported (Ikeuchi et al., 1996). This effect resembles the dual action of FFA on Na^+ channels described in previous sections. Pretreatment with PKC inhibitors did not counteract the FFA inhibition but abolished the potentiation effect. However, experiments performed with mutant nAChR lacking PKC phosphorylation sites showed no current blocking effect of AA and a current potentiation greater than in the control condition. Together, these results pointed to two different and independent mechanisms: a short-term blocking mechanism through direct action of AA on nAChR PKC phosphorylation sites, rather than through an effect on the membrane environment; and a long-term potentiating mechanism caused by PKC activation not involving nAChR phosphorylation. This second mechanism must include an unknown pathway related to AA-regulated PKC. Different results were observed depending on the FFA structure. Treatment with linoleic (18:2) or linolenic (18:3) acids caused similar dual effects on *Torpedo* nAChR; however, the long-term potentiation was not evident in mutant nAChR, indicating that these FFA, in contrast to AA, did in fact potentiate nAChR via its PKC phosphorylation site (Nishizaki et al., 1997a). On the other hand, current potentiation by treatment with oleic acid (18:1) was not hindered by PKC inhibitors but fully inhibited by KN-62, a calmodulin-dependent protein kinase II (CaMKII) inhibitor, suggesting that oleic acid enhances nAChR currents by activation of CaMKII, independently of the PKC pathway (Nishizaki et al., 1997b). This can be accounted for by the explanation provided by Gérczy et al. (2012), pointing to the AA selectivity of certain PKC isoforms over several other signal-transduction mechanisms. Lysophosphatic acid also enhances *Torpedo* nAChR currents both in control and in mutant nAChRs lacking PKC phosphorylation sites; however, the potentiation was also completely blocked by GF109203X (Nishizaki and Sumikawa, 1997). These authors suggested that lysoPA probably potentiates nAChR currents by pertussis toxin-insensitive G-protein and activation of Ca^{2+}-dependent/-independent PKCs with subsequent phosphorylation of the receptors and, additionally, by an unknown factor or process activated by PKC activation. Saturated FFA with less than 20 C atoms also potentiate nAChR currents with the participation of PKC, stearic acid (18:0) being the most potent FFA in this respect (Ohta et al., 2003). A comprehensive study of the importance of the FFA structure (Yaguchi et al., 2005) concluded that FFA modulation of nAChR current could not be simply explained by the number and position of the *cis*-double bonds. This study showed that 20:1ω12 (**8**-eicosenoic acid) potentiated the currents without depression, whereas 20:1ω15 (**5**-eicosenoic acid) and 20:1ω9 (11-eicosenoic acid) elicited only the depression component, suggesting that *cis*-double bond at the 8th position plays a role in the potentiation and at the 5th or 11th position in the depression. This rule agrees with the fact that 20:3ω9 (5,8,11-eicosatrienoic acid), 20:4ω6 (5,8,11,14-eicosatetraenoic acid), and C20:5ω3 (5,8,11,14,17-eicosapentaenoic acid) induced a transient inhibition followed by enhancement of the currents, whereas 20:3ω6 (8,11,14-eicosatrienoic acid) induced only nAChR depression and not potentiation (Yaguchi et al., 2005). It should be noted, however, that 20:2ω6 (11,14-eicosadienoic acid) has no double-bonds at crucial positions and it has a dual effect on nAChR currents. The carboxy-terminal group appears to play a critical role in the potentiation of the nAChR, as this group is necessary for PKC-ε activation; replacement of COOH in linoleic acid by CONH2 (linoleoylamide) only caused the depression effect (Yaguchi et al., 2005).

The work of Vijayaraghavan et al. (1995) was the first demonstration that FFA also modulate neuronal-type nAChRs. Using chick ciliary ganglion neurons these authors showed that seconds of incubation with AA sufficed to inhibit neuronal nAChR with a distinct and largest effect on the α7-type nAChR. This inhibition was not restricted to AA but also included other FFA in a structure-dependent manner: saturated or cis/trans monounsaturated FFA showed little or no effect; FFA having two or three double bonds exhibited inhibitory

TABLE 1 | Summary of effects produced by different FFAs on the nAChR (Perillo et al., 2012).

	 Petroselenic acid (18:1 *cis*-**6**)	 Oleic acid (18:1 *cis*-**9**)	 Vaccenic acid (18:1 *cis*-**11**)	 Octadecanoic acid (18:1 *cis*-**13**)	 Elaidic acid (18:1 *trans*-**9**)
Sites at lipid-nAChR interface	A[a]	A + NA[b]	A + NA	A + NA	A + NA
inhibition of nAChR function	+	++	−	−	−
GP modification	++	+++	++	+	−
Anisotropy modification	+	+++	++	++	−
nAChR TM4 perturbation	+	++	++	++	−
R-state perturbation	−	++	++	++	−
D-state perturbation	++	++	+	+	+

The CPK structures of the FFAs studied are shown for clarity: the broken line shows the position of the cis double bond and the arrow indicates the position of the trans double bond.
[a]A, annular sites; [b]NA, non-annular sites.

effects, and AA displayed maximal effect. The authors concluded that the inhibition must be caused by a direct action of the FFA on the nAChR or through the membrane, discarding the possible action of AA metabolites or the activation of PKC. Minota and Watanabe (1997) also showed that AA directly inhibits nAChR in bullfrog sympathetic ganglia, and that its metabolites do not play a major part in this inhibition. Two general possibilities were postulated to account for this mechanism: (i) AA binds to allosteric sites of the nAChR –one possibility being a site in the channel pore- thus inhibiting synaptic transmission without affecting the binding of ACh to the receptor; or (ii) AA perturbs the local environment of the receptors by partitioning into the membrane and thereby indirectly inhibiting receptor function. Nishizaki et al. (1998) indicated that neuronal nAChRs display short-term depression and/or long-term enhancement of nAChR currents depending on the biological source of the neuronal nAChR. Treatment of chick α7 nAChR with AA caused only a depression effect (in accordance with Vijayaraghavan et al. (1995)) whereas in the case of rat α7 nAChR the same treatment caused exclusively a potentiation effect by PKC activation. This strongly suggests a relation between FFA effect and nAChR structure; the different response to AA may probably be due to structural differences between receptors. Further studies with rat α7 and α2β4 nAChR showed that AA increases glutamate release by potentiating the activity of presynaptic nAChRs, predominantly α7 nAChR, under the influence of PKC, an effect not related to CaMKII activity (Nishizaki et al., 1999). Stearic acid has also been shown to potentiate α7 nAChR currents, with the involvement of PKC in this potentiation (Ohta et al., 2003). The latter work demonstrated that although stearic acid enhances activity of already active PKC-ε it does not directly activate this enzyme.

Studies of the FFA modulation of different proteins through PKC indicate that, in some cases, the mechanism involves not a direct PKC activation but an increased expression and altered subcellular distribution of PKC. It is known that translocation of PKC isoforms is generally regarded to be indicative of their activation. Li et al. (2010) explained the induced pulmonary artery (PA) contraction by hypoxia through an important increment in both the RNA levels and protein expression of PKC-δ and PKC-ε induced by 15-hydroxyeicosatetraenoic acid (HETE). In this case, AA is metabolized by 15-lipoxygenase, which is up-regulated by hypoxia, to 15-HETE which causes pulmonary artery (PA) constriction by activation of PKC-δ and PKC-ε. A similar profile was described in HEK293 cells, where AA displays a biphasic effect on Ca^{2+} signaling: at low concentrations, AA suppresses both Ca^{2+} release and Ca^{2+} influx responses to agonist; whereas at high concentrations, AA potentiates Ca^{2+} release and Ca^{2+} entry response (Chen et al., 2012). These authors postulated that AA induces PKCα and PKCβII redistribution at the plasma membrane, and that at higher AA concentrations trafficking of PKCβI and PKCβII to the endoplasmic reticulum also occurred. A totally opposite effect was described in a study of the immunomodulatory properties of PUFA in phagocyte function (Gorgani et al., 2011). These authors demonstrated that AA causes a significant decrease in CRIg expression at both the mRNA and protein levels, and that this down-regulation is dependent on PKC activation by AA excluding the possibility that AA could exert its effect through its metabolism via cyclooxygenase and/or lipoxygenase.

It is important to highlight that the FFA concentrations used in all the studies mentioned here were below the high FFA levels reported in several pathological conditions as a consequence of PLA2 activation and FFA release from plasma membranes. Arachidonate levels in the plasma of malaria patients are in the

order of 100 μM (≈ 33.3 μg·mL^{-1}) (Eissen, 1993) and under ischemic conditions these levels can rise to 500 μM, 10-fold higher than the free AA levels found in normal brain (Yasuda et al., 1985). A 10-fold increment in FFA was also reported in an experimental model of acute lung injury (Arbibe et al., 1998).

More recent studies used FA derivatives to explore in more detail the modulatory mechanism exerted by FFA on α7 nAChR. FR236924, a linoleic acid derivative, was synthesized having cyclopropane rings instead of *cis*-double bonds. The FA analog induced a long-lasting facilitation of hippocampal neurotransmission, as assessed by the persistent enhancement in the activity of presynaptic nAChRs via a PKC pathway (Tanaka and Nishizaki, 2003; Yamamoto et al., 2005). 4-[4-(Z)-hept-1-enyl-phenoxy] butyric acid (HUHS2002) potentiated rat α7 nAChR currents, an effect that was not affected by the addition of an inhibitor of PKC but significantly inhibited by an inhibitor of CaMKII (Kanno et al., 2012a). This suggests that HUHS2002 potentiates α7 nAChR currents by activation of CaMKII. However, HUHS2002 might indirectly activate CaMKII by inhibiting protein phosphatase 1 (PP1), which normally dephosphorylates and inactivates CaMKII. Another linoleic acid derivative with cyclopropane rings instead of *cis*-double bonds (DCP-LA) preserved the potentiation effect on nAChR currents, which was abolished by GF109203X, a PKC inhibitor (Kanno et al., 2012b). The DCP-LA effect (a) was significantly inhibited by vesicular transport inhibitors, (b) promoted the translocation of the α7 nAChR from the cytosol to the plasma membrane and (c) stimulated α7 nAChR delivery toward presynaptic terminals. The evidence suggests that the nAChR current potentiation could arise from the DCP-LA mediated stimulation of nAChR vesicular transport and the consequent increase in the number of nAChR targeted to the cell surface (Kanno et al., 2012b). This was the first postulation of PKC control of intracellular α7 nAChR trafficking; which PKC targets participate in this regulation is still not known. Subsequent studies showed that DCP-LA significantly increased an association of 4.1N -a scaffolding protein- with α7 nAChR; and that this association is partially prevented by GF109203X, an inhibitor of PKC, but independently of 4.1N phosphorylation (Kanno et al., 2013). The same study showed for the first time that 4.1N is required for translocation of the α7 nAChR toward the plasma membrane. Thus, DCP-LA could induce an increase in the association of the α7 nAChR with 4.1N in a PKC-dependent manner, not caused by PKC phosphorylation of 4.1N or by phosphorylation of the receptor. Probably DCP-LA, by activating PKC, phosphorylates an unknown factor that enhances the association of 4.1N with the α7 nAChR (Kanno et al., 2013). Thus, the evidence described in the last paragraphs point to a novel pathway linking lipid signaling to α7 nAChR responses, as is graphically summarized in **Figure 3**.

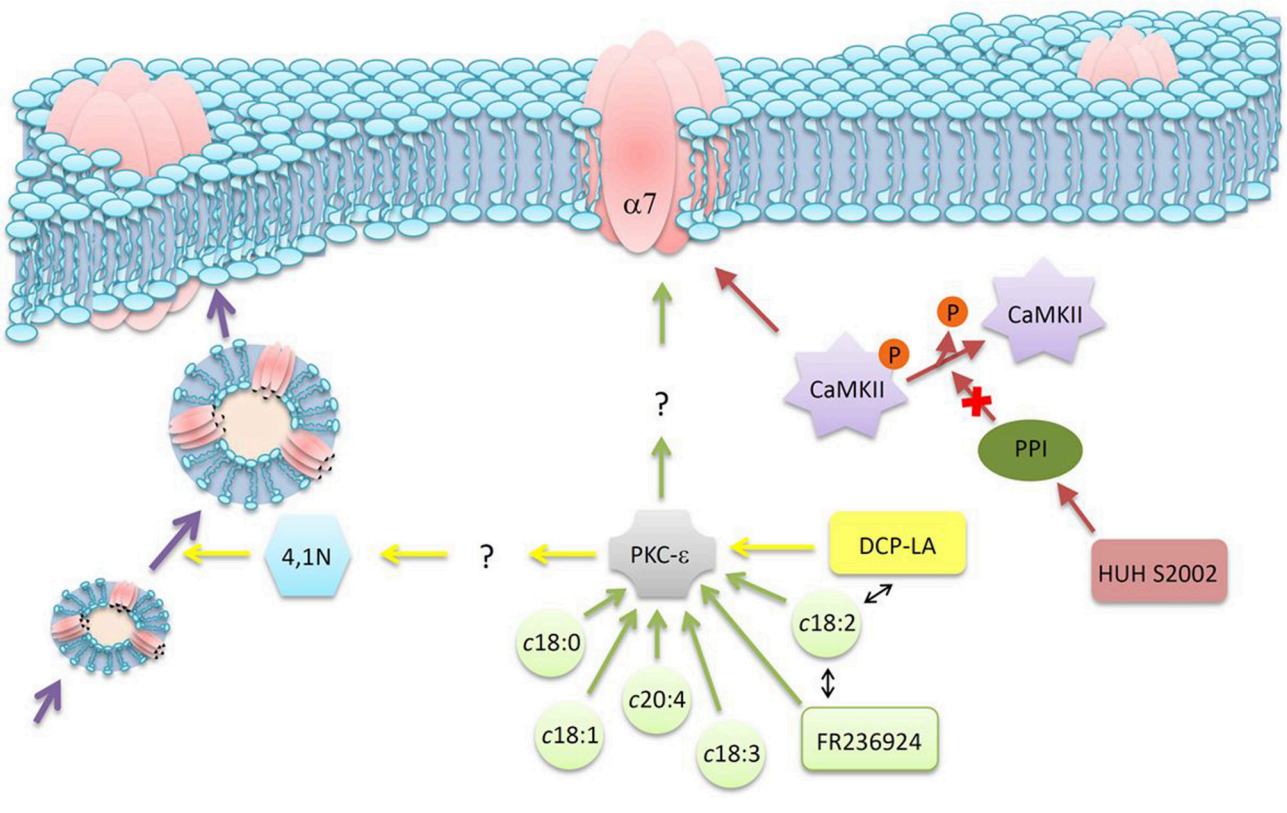


FIGURE 3 | Schematic diagram of indirect mechanisms of action of FFA on neuronal nAChR function. Different FFA, or their derivatives, can modulate α7 nAChR through various signaling pathways. See text for details.

One avenue that needs to be explored in greater detail is the involvement of abnormalities of FA metabolism in brain diseases. Circulating long-chain fatty acids act as signals of nutrient surplus in the hypothalamus. Furthermore, pharmacological and/or genetic inhibition of FA synthase, AMP-activated protein kinase and carnitine palmitoyl transferase 1 (CPT1) results in marked decreases in feeding levels and loss of body weight in rodents (López et al., 2007). CPT1c, the recently discovered brain isoform of the multiprotein complex enzyme carnitine palmitoyl transferase, is predominantly localized in regions involved in the regulation of food intake, such as the hypothalamus, emotion and reward systems -the amygdala- and learning and memory, the hippocampus. CPT activity has been associated with dysregulation of insulin equilibrium in brain and related metabolic dysfunctions, and implicated in the evolution of Parkinson's and Alzheimer's diseases (Virmani et al., 2015).

CONCLUSIONS

When analyzing the effects of FFA on a variety of ion channels, one of the first clear outcomes is that different FFA exert distinct effects, or no effect at all, on a same channel protein, whereas similar FFA may cause diverse effects on different proteins, even if the latter are very closely related from a phylogenetic point of view. In spite of these apparent discrepancies, it is clear that almost all FFA that modulate the ion channel directly, i.e., not through their metabolites or signaling cascades, act *by direct physical contact with the protein.* Direct mechanisms consequently put both FFA structure and channel structure - particularly those amino acids that participate in the interaction- at center stage. Amino acid residues involved in the recognition of FFA are apparently sensitive to the length, isomerism and saturation of the FFA. It is thus mandatory to identify and characterize in detail the structure of the intervening binding site(s) in the protein and the counteracting FFA structure to unravel the different inhibitory or stimulatory modalities at the molecular level and eventually contribute to the design of new lipid-based modulatory drugs targeting specific channel proteins.

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REVIEW
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In-Depth Study of the Interaction, Sensitivity, and Gating Modulation by PUFAs on K^+ Channels; Interaction and New Targets

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Voltage gated potassium channels (K_V) are membrane proteins that allow selective flow of K^+ ions in a voltage-dependent manner. These channels play an important role in several excitable cells as neurons, cardiomyocytes, and vascular smooth muscle. Over the last 20 years, it has been shown that omega-3 polyunsaturated fatty acids (PUFAs) enhance or decrease the activity of several cardiac K_V channels. PUFAs-dependent modulation of potassium ion channels has been reported to be cardioprotective. However, the precise cellular mechanism underlying the cardiovascular benefits remained unclear in part because new PUFAs targets and signaling pathways continue being discovered. In this review, we will focus on recent data available concerning the following aspects of the K_V channel modulation by PUFAs: (i) the exact residues involved in PUFAs-K_V channels interaction; (ii) the structural PUFAs determinants important for their effects on K_V channels; (iii) the mechanism of the gating modulation of K_V channels and, finally, (iv) the PUFAs modulation of a few new targets present in smooth muscle cells (SMC), $K_{Ca}1.1$, K_{2P}, and K_{ATP} channels, involved in vascular relaxation.

Keywords: PUFAs, shaker K channels, Kv7.1, K2P, KCA, KATP, lipoelectric hypothesis

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INTRODUCTION

It has been reported that an increased consumption of omega 3 polyunsaturated fatty acids (n-3 PUFAs; **Figure 1**) have beneficial properties for the cardiovascular system (Chaddha and Eagle, 2015) and neurological diseases such as epilepsy and pain (Lefevre and Aronson, 2000). Among other targets, the beneficial actions of n-3 PUFAs occur through the modulation of a big variety of K^+ voltage gated ion channels (Boland and Drzewiecki, 2008; Moreno et al., 2012). For instance, it is known that n-3 PUFAs activate different members of the K_V7 channel family. In the heart, n-3 PUFA dependent activation of $K_V7.1$ channels (pore forming component of the cardiac I_{Ks}) reduces the risk of arrhythmia by shortening the action potential duration (Verkerk et al., 2006; Liin et al., 2015; Moreno et al., 2015). In the neuronal system, the activation of $K_V7.2/K_V7.3$ channels (major components of the neuronal M-current) decreases neuronal excitability and therefore the risk of seizure (Liin et al., 2016; Valenzuela, 2016).

Despite the encouraging results of the first clinical trials published in the early 2000s about n-3 PUFAs protective effects, very soon other studies reported no beneficial or even harmful effects. For example, an increased risk of cardiac events was found in angina pectoris patients

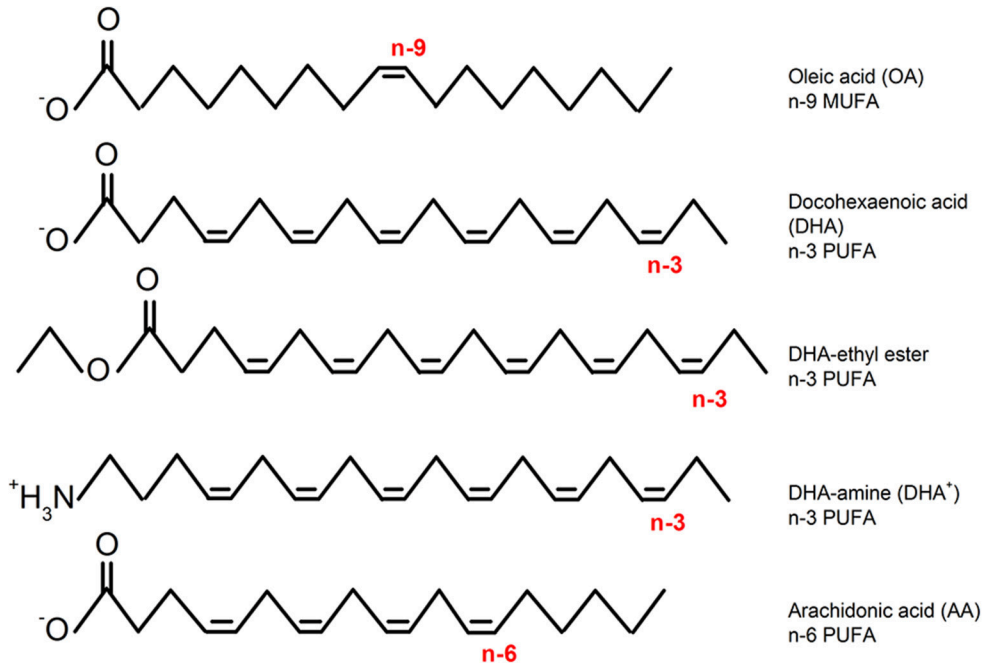

FIGURE 1 | Chemical structures of representative fatty acids (monounsaturated: n-9 MUFA, n-3, and n-6 PUFAs) and modified PUFAs (DHA-ethyl-ester DHA and DHA-amine: non-charged and permanently charged, respectively).

(Burr et al., 2003). The physiological effects of PUFAs are complex and poorly understood. In the context of excitable diseases, we believe that the conflicting results emerged from the lack of exhaustive knowledge on two aspects of the n-3 PUFA-dependent modulation of voltage-gated ion channels. The mechanisms by which n-3 PUFAs differentially modulate ion channels and for which specific pathologies n-3 PUFAs are truly beneficial are poorly understood. Different cardiovascular and neuronal pathologies are triggered by different underlying mechanism. Therefore, a diet rich in n-3 PUFAs might be beneficial for some conditions but neutral or deleterious for others.

The mechanism of action by which PUFAs modify ion channel activity has been debated for years. Several theories, ranging from (1) non-specific effects on the cell membrane (Leifert et al., 1999) to (2) specific binding to the ion channel (Hallaq et al., 1992; Kang and Leaf, 1996; Bendahhou et al., 1997; Xiao et al., 2005; Guizy et al., 2008), have been proposed. Scientific evidence in favor of the indirect modulation of ion channels by n-3 PUFAs are the lack of selectivity in blocking a given channel *vs.* other, and the reported effects on the cell membrane fluidity. On the other hand, the identification of residues in the ion channel protein involved in n-3 PUFAs sensitivity argues in favor of the second theory. A great step forward defining the molecular mechanism of n-3 PUFAs modulation of K_V channels has been recently achieved. The identification of the PUFA binding site on several K^+ channels, the proposed mechanism of action for the direct effects of PUFAs and the experimental evidence showing that n-3 PUFAs can modify the gating of ion channels simultaneously by establishing direct interactions and by modifying the properties of the lipid bilayer were essential findings that helped to clear up the controversy (Borjesson et al., 2008; Moreno et al., 2015).

Important advances have been achieved in the identification of the specific pathologies that could benefit from a diet rich on n-3 PUFAs as well. In this regard, many new ion channel targets in different organs and tissues have been identified, and disease animal models have been produced to understand how PUFAs can affect physiology.

In the present review we first summarize results identifying the PUFA binding site on K^+ ion channels, the molecular determinants that determine the affinity and potency within the n-3 PUFA molecule, and the proposed mechanism of action by which n-3 PUFAs modify the gating K^+ channels: the "lipoelectric hypothesis." In the second part of the review, we focus on recent findings in PUFAs-dependent modulation of the K_V7 family and a few new targets: K_{2P}, $K_{Ca}1.1$, and K_{ATP} in the cardiovascular and neuronal function. Finally, we briefly discuss how the PUFAs represent a new pharmacological treatment in which ion channel voltage dependence is electrically modulated instead of the more traditional pore block.

SELECTIVE PUFA EFFECTS ON DIFFERENT POTASSIUM CHANNELS

In this section we recapitulate the arguments in favor of specific PUFA-ion channel interactions emphasizing findings published in last 5 years. We will focus on two potassium channels *Shaker* and $K_{Ca}1.1$, for which the PUFA induced modulation of ion channels, has been more extensively characterized.

Evidences for Selective PUFAs Effects on *Shaker* K^+ Channels

After the initial studies based on the introduction of point mutations in the ion channel to demonstrate point direct interaction between the ion channel and the PUFA (Xiao et al., 2001a,b), another solid evidence showing how PUFAs differently modify ion channels raised from the observation that the potency of the n-3 PUFA docosahexaenoic acid (DHA) on *Shaker* voltage dependence changes with local pH (Borjesson et al., 2008). At physiological pH, DHA increased *Shaker* K^+ current by shifting the midpoint of activation [G(V) curve] toward hyperpolarized potentials. It is known that different ion channels have different local pH values depending on their local set of surface charges structure (Elinder et al., 1996). To explore the influence of the surface charges and local pH on *Shaker* modulation by DHA, Borjesson and colleagues used a mutated *Shaker* channel where residues A419, F425, and V451 were made positive (Borjesson et al., 2008). The presence of these three positive residues in the extracellular loops connecting transmembrane segments S5 and S6 of *Shaker* K^+ channels resulted in a local pH change of 0.3. Under these conditions, DHA induced a negative shift of the midpoint of activation in the triple mutant about twice as large as the shift induced in the WT *Shaker* K^+ channel. These results clearly indicate that the PUFA-induced effect is channel specific and depends on the channel-specific set of surface charges (Borjesson et al., 2008).

The ultimate proof for the specific PUFAs effects on different ion channels came with the identification of the PUFAs binding site on *Shaker* channels. Because of its lipophilic character and modulatory effects it was proposed that the PUFAs binding site on *Shaker* K^+ channels should be in the vicinity of the gating charges in the voltage sensor (S4 segment) and near residues facing the lipid bilayer (Borjesson et al., 2008). The confirmation for the proposed binding site came quickly. A cysteine scan analysis covering the lipophilic surfaces of the extracellular halves of S3–S6 segment showed that residues I325C and T329C located in the carboxyl end of helix S3, and I360C at S4 were insensitive to DHA. On the contrary, the L366C mutation increased DHA sensitivity of *Shaker* K^+ channels (Borjesson and Elinder, 2011). To further define the PUFAs binding site, positives charges were introduced to each of the above-mentioned residues in the S3–S4 regions by using $MTSEA^+$ reagent. Consistently with the cysteine scan data, a positive charge at residues I325; T329 and A359; I360 of the S3 and S4 respectively resulted in an increased sensitivity to DHA effects (Borjesson and Elinder, 2011). In addition to the experimental data, a structural model built to predict 3D interactions suggested that a negative charge at R1 (R362) would reduce the PUFA effect. Consistently with the model, when the charge of R362C mutant was modified negatively with $MTES^-$ reagent the G(V) shift induced by DHA was smaller than that induced in WT *Shaker* K^+ channels. In contrast, $R362C^+$ (exposed to $MTSET^+$) restored PUFA sensitivity. Some K_V channels such as $K_V1.2$ have an additional gating charge R0 at the top of the S4 segment. An homology model of the *Shaker* K^+ channel based on the $K_V1.2/2.1$ chimera predicts that a positive residue at that position (A359) could strengthen the interaction between the PUFA head group and the ion channel (Borjesson and Elinder, 2011). When attaching $MTSET^+$ to A359C, the DHA-induced G(V) shift was greater. Experiments in which the charge of R0 and R1 was changed support the proposed localization of the PUFA binding site and suggested that different PUFAs should have very different effects in modulating different ion channels depending on the presence of a charge at positions R0 and/or R1 (Borjesson and Elinder, 2011). Together, the data showed that high impact residues for DHA on *Shaker* K^+ channels are clustered in a small region of the lipid facing S3–S4 corner of the voltage sensor domain but distant from the pore region (**Figure 2**). Positive charges located close to the PUFAs binding site increase PUFA potency.

As shown for *Shaker* K^+ channels, it has been reported that PUFAs target the voltage sensor domain of many other K_V channels such as $K_V7.1$ channels (Liin et al., 2015). In $K_V7.1$ channels, PUFAs interact with extracellular positively charged residues of the S4 segment to promote channel opening. Neutralization of the most external gating charge (R1) of the S4 of $K_V7.1$ channels, R228Q, made $K_V7.1$ channels insensitive to DHA, to DHA-glycine (a permanently deprotonated DHA

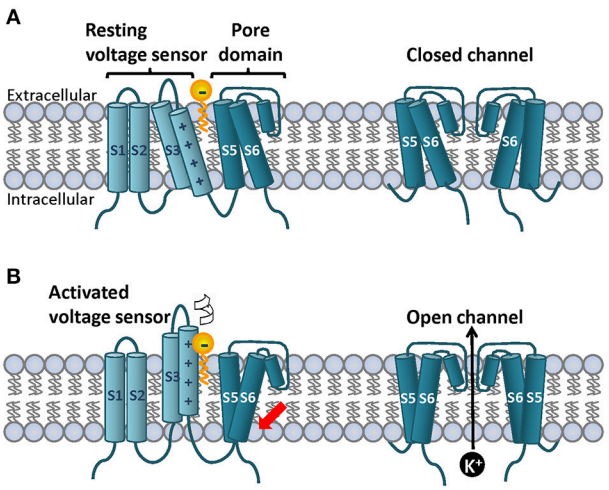

FIGURE 2 | Topology of a canonical voltage gated ion channel subunit on the lipid bilayer and cartoon representation of the lipoelectric hypothesis mechanism of PUFA-dependent modulation of *Shaker*-like K^+ channels. On the cell membrane four subunits co-assemble to for the ion channel. **(A)** Each subunit contains six transmembrane segments and N-and C-terminal domains. S1–S4 segments form the voltage sensor and S5–S6 form the ion pore. The S4 segment contains a variable number of positively charged residues known as gating charges that detect small changes on the electric field on the membrane. In response to prolong depolarizations the S4 moves upward perpendicular to the membrane and rotates. **(B)** This process is called voltage sensor domain activation and it is indicated by the white arrow. Once the four individual S4 are on the upstate the activation gate located at the base intracellular site of the pore domain can open to allow K^+ flow (red arrow). A simplified PUFA structure is depicted with the head group located at the extracellular bilayer interface and the bilayer center is depicted in yellow. The PUFA head group interacts electrostatically with the upper gating charges of the S4 promoting mainly channel opening (red arrow). Top and bottom right panels show the front view of the S5 and S6 segments in the closed and open state. Two subunits are depicted for simplicity.

analog at physiological pH due to its lower pKa value) and to N-arachidonoyl taurine (with a permanent negative charge at pH = 7.4). Besides the S4 gating charge residues, other amino acids located in the outer halves of S3 and S4 had a big impact in the PUFA modulatory effect. Again a cysteine scan analysis showed that residues K218C and G219C, located in the S3–S4 extracellular linker of $K_V7.1$ reduced the PUFA effect by a factor of 3, suggesting the existence of PUFA binding site equivalent in other K_V channels (Liin et al., 2015).

Evidences for Selective PUFAs Effects in $hK_{Ca}1.1$ Channels

Although in general different potassium channels show high similarity regarding structural domains and gating mechanisms, some important differences exist between different K_V families. One proof of that is the different location of the PUFA binding site on different K_V families as demonstrated for $hK_{Ca}1.1$ channels (or large conductance Ca^{2+}- activated K^+ channels).

In a study by Hoshi and colleagues it was shown that DHA increased $hK_{Ca}1.1$ channels by 2.5-fold and induced a shift of the G(V) curve into the negative direction (Hoshi et al., 2013c). Regardless the potentiating effects of DHA on $hK_{Ca}1.1$ channels $K_{Ca}1.1$ channels from Drosophila were insensitive to DHA. In order to determine the molecular determinants within $hK_{Ca}1.1$ channels critical for the stimulatory effect, chimeric constructions of Human/Drosophila $hK_{Ca}1.1$ channels were done. The chimeric constructions revealed that the PUFA binding site is located in the pore domain (S5, P-loop and S6 segments) of $hK_{Ca}1.1$ channels. Furthermore, the introduction of Human-to-Drosophila point mutations in the pore domain of $hK_{Ca}1.1$ highlighted the residue Y318S (located in the ion pore) as a crucial determinant of the differential sensitivity of $hK_{Ca}1.1$ channels compared to $dK_{Ca}1.1$ to PUFAs (Hoshi et al., 2013c).

Besides the identification of the PUFA binding site in $K_{Ca}1.1$ channels, additional evidence in favor of a selective action of PUFAs in $hK_{Ca}1.1$ channels raises from the different potency shown by the fatty acids in $hK_{Ca}1.1$ channels co-expressed with different modulatory subunits. In different tissues, $hK_{Ca}1.1$ channels are co-assembled with β1, β2, β4, or ϒ subunits (Wu and Marx, 2010). Another study performed by Hoshi and colleagues, showed a 2.5-fold current density increase induced by DHA on $hK_{Ca}1.1$ channels (expressed in the absence of modulatory subunits), while it increased $hK_{Ca}1.1$+β1 and $hK_{Ca}1.1$+β4 by up to 20-fold and shifted the G(V) by −60 mV. On the contrary, DHA was much less effective in $hK_{Ca}1.1$+β2 and $hK_{Ca}1.1$+ϒ1 channels (**Figure 3**). These differential effects are very noteworthy because all β subunits share a similar amino acid sequence and structural organization. Chimeric experiments determined that the N-terminus and the first transmembrane domain of β1 and β4 subunits are crucial for DHA sensitivity. Finally, point mutations experiments in which amino acids in β1 or β4 were substituted with that in β2 at the corresponding location revealed that R11/E12 and C18/R19 are crucial for DHA sensitivity in β1/β4 respectively. In addition, β2–β1 or β4 substitutions at the equivalent positions were made which showed indistinguishable sensitivity to DHA compared to β1 and β4 subunits (Hoshi et al., 2013a). All these results may suggest that PUFAs binding site resides in both the α and in the β1 and β4 subunits of the $hK_{Ca}1.1$ ion channel complex.

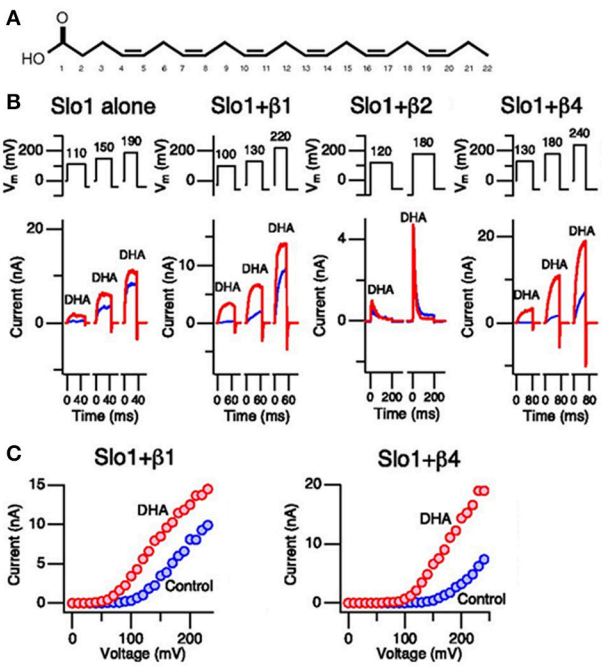

FIGURE 3 | Effects of DHA on $K_{Ca}1.1$ channels. (A) Chemical structure of DHA. **(B)** Currents generated by $K_{Ca}1.1$, $K_{Ca}1.1$+β1, and $K_{Ca}1.1$+β4 channels in inside-out patches recorded in the absence of calcium. Current records under control conditions and after addition of DHA (3 μM) are shown in blue and red, respectively. **(C)** Current-voltage relationships obtained from $K_{Ca}1.1$+β1 and $K_{Ca}1.1$+β4 are shown. Again, results from control and DHA are shown in blue and red, respectively. Hoshi et al. (2013a)

PUFAS STRUCTURAL DETERMINANTS IMPORTANT FOR THEIR EFFECTS ON ION CHANNELS

In parallel to the progression in the understanding of the molecular determinants within the ion channels involved in interactions with PUFAs, several publications have contributed to the identification of the features within the PUFAs molecules important for their effects. The PUFA molecule consists of an aliphatic chain (of variable length) with two or more double bonds capped at the end with a negatively charged carboxyl group. Which are the crucial features for the modulation of K^+ channels?

The Charge of the Carboxyl Group is Crucial for the Electrostatic Interaction with the Voltage Sensing Domain of the Ion Channel

PUFAs such as DHA and arachidonic acid (AA) are negatively charged at physiological pH 7.4. In order to test the effect of the charge of the carboxyl group, Borjesson et al. (2008)

compared the effects of DHA (negatively charged), DHA methyl ester (non-charged transesterified DHA), and a charged lipolytic toxin GsMTx4$^+$ (structurally similar to PUFAs but with four positive charges) on *Shaker* channels. DHA shifted the G(V) curve by 10 mV toward the negative potentials. DHA methyl ester had no effect on the voltage dependence. Conversely, the GsMTx4$^+$ toxin shifted the G(V) curve 5.7 mV into the positive direction. In the same line of evidence it was shown that the potency of DHA changes with pH. A higher pH potentiates DHA effects because a larger fraction of the PUFA is deprotonated (Borjesson et al., 2008). In another study, the pH-dependence of the modulation of *Shaker* by arachidonoyl amine (AA$^+$) was analyzed (Borjesson et al., 2010). AA$^+$ shifted the G(V) curve of *Shaker* K$^+$ channels toward positive potentials and decreased the maximum conductance. AA methyl ester had no effect. Following a similar reasoning, if there is a pH-dependence for the amine effect AA$^+$ should be more effective at low pH. As expected, AA$^+$ was more potent at a lower pH when a larger fraction of AA$^+$ is protonated (Borjesson et al., 2010).

The importance of a negative charge on the head group of DHA is applicable to many other PUFAs but also to many other potassium channels. In a recent study by Liin et al. (2015), it was shown that DHA (negatively charged) shifted the G(V) by ~ -9 mV of K_V7.1 channels. On the contrary, DHA$^+$ and AA$^+$ shifted the G(V) by $\sim +9$ mV. Together, these results demonstrate that the modulation of the voltage dependence of activation of K$^+$ channels toward hyperpolarized or depolarized potentials is determined by the valence of the charge in the carboxyl head group. A negative charge shifts the G(V) curve toward more negative potentials and vice versa.

Two or More Cis Double Bonds in the Acyl Chain are Necessary to Shift G(V) Curve

Borjesson and colleagues tested ten fatty acids or fatty acid-like compounds with different acyl chain lengths and different numbers, position, and geometry of double bonds to identify structural features on the acyl tail important for the PUFA modulation of *Shaker* K$^+$ channels. Fatty acids with the most structural similarity to DHA by having two or more methylene-interrupted double bonds at either the n-3 or the n-6 position showed similar shifts of the G(V) curves in *Shaker* channels (Borjesson et al., 2008). The rest of the compounds were not effective.

Recently, Yazdi et al. (2016) studied how the saturation levels in the carbon tail affect the structure of the lipids in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane patches. From their molecular dynamics simulations the following conclusions were extracted: (i) the order parameters of PUFA and saturated fatty acids decreased from the carboxyl end located in the bilayer interface to the end of the acyl chain tail in the bilayer center. (ii) The order parameters displayed a different overall shape reflecting the positions of the cis double-bonds of PUFAs. (iii) There are differences in the shape and packing properties by measuring the radius of gyration of the carbon tail and head-to-tail length. Compared to other fatty acids, the PUFA molecule tends to twirl and curl up. All together, these results indicate that the PUFA molecule has greater conformational mobility compared to saturated fatty acids. The predominant saturated fatty acid conformation is more extended resulting in a decreased flexibility and mobility of phospholipids of the cell membrane (Yazdi et al., 2016).

Effect of the n-3 PUFA Acyl Chain Length

Contrary to the importance of a negatively charged carboxyl group for the PUFA effect and the presence of two or more double bonds, the impact of the length of the acyl tail for the PUFAs effects is not so obvious. In *Shaker* K$^+$ channels the presence of two or more double bonds and its location in cis geometry (more flexible) seem to be more important than the number of carbons of the tail for the PUFA induced G(V) shift (Borjesson et al., 2008). Similar findings were shown by Liin et al. in K_V7.1 channels (Liin et al., 2015). Two fatty acids: EPA and oleic acid (OA), poly- and mono-unsaturated fatty acids respectively with similar acyl tail length showed very different effects on K_V7.1 currents. OA had no effect on K_V7.1 currents while EPA showed a similar potency increasing K_V7.1 compared to DHA (Liin et al., 2015). On the other hand, a study by Hoshi et al. (2013c) in which the effects of three n-3 PUFAs: DHA, EPA and α-linolenic acid (ALA) were examined in $hK_{Ca}1.1$ channels revealed that DHA had the largest stimulatory effect on $hK_{Ca}1.1$ currents followed by EPA and finally ALA. These results suggest the importance of the length of the acyl might be channel specific and that the longer the acyl tail, the larger the effect of the PUFA will be.

MECHANISM OF GATING MODULATION: LIPOELECTRIC HYPOTHESIS

Origin of the Lipoelectric Hypothesis and Definition

Based on the following effects of DHA on *Shaker* K$^+$ channels: (i) shift of the G(V) and of the steady state inactivation curve to the negative direction, and (ii) acceleration of the activation kinetics and slower deactivation kinetics; Borjesson and colleagues formulated in 2008 the "Lipoelectric hypothesis" to explain the PUFAs-dependent modulation of K$^+$ channels mechanism. All these findings are in agreement with DHA and other PUFAs causing electrostatic effect on the voltage sensor of ion channels (Borjesson et al., 2008). The lipoelectric hypothesis proposes that the lipophilic PUFAs bind to a hydrophobic environment (the lipid bilayer or bilayer/channel interface close to the S3-S4 segment) from where they act electrostatically on the voltage sensor changing the voltage dependence of the ion channel (Borjesson et al., 2008).

Which Activation Steps are Affected by PUFAs?

It is known that the activation of *Shaker* K$^+$ channels occur in at least two steps. In the early transitions the four *Shaker* subunits go through transitions between the closed states (C0 to C2) and, then the channel goes through other two transitions to the final closed and the open states (C2 to O). The transitions

between the first closed states are sequential and represent the activation of the voltage sensor, whereas those between C2 to O are concerted and couple the movement of the main voltage sensors to channel opening (Schoppa et al., 1992; Bezanilla et al., 1994; Zagotta et al., 1994; Schoppa and Sigworth, 1998).

$$\begin{array}{ccccccccccccc} & \alpha_0 & & \alpha_1 & & \alpha_1 & & \alpha_1 & & \alpha_2 & \alpha_3 & \alpha_4 & \\ C0 & \leftrightarrow & C1 & \leftrightarrow & C11 & \leftrightarrow & C12 & \leftrightarrow & C2 & \leftrightarrow C3 & \leftrightarrow C4 & \leftrightarrow & O \\ & \beta_0 & & \beta_1 & & \beta_1 & & \beta_1 & & \beta_2 & \beta_3 & \beta_4 & \end{array}$$

To explore which activation step is affected by DHA, gating currents were measured in two mutated *Shaker* K^+ channel constructions. The final transitions (from close C2 to open O) were studied in the ILT mutant *Shaker* channel where three hydrophobic residues in S4 are substituted for three other hydrophobic residues (V369I, I372L, and S376T). In the ILT mutant the voltage sensor activation and pore domain opening steps are energetically separated allowing the study of the late transitions during the channel activation in isolation The initial steps of activation (C0 to C2) were studied in the non-conducting channel resulting from the ILT mutant plus the pore mutation W343F. DHA significantly shifted the G(V) curve in the negative direction along the voltage axis. On the contrary, DHA had a much smaller effect on the gating currents Q(V) of the ILT/W343F *Shaker* mutant. The larger effect of DHA seen on the G(V) compared to the Q(V) curve suggests that the opening step (the second gating component) is more affected than the early transition by the PUFA (Borjesson et al., 2008). Despite DHA had a much smaller effect on the gating currents, this small effect indicates that the activation of independent voltage sensors is also affected. A gating model of *Shaker* K^+ channels predicted a DHA-dependent shift of 30 and 5 mV in the G(V) and Q(V) respectively corroborating the experimental data (Borjesson and Elinder, 2011).

PUFA Head Group is Closer to *Shaker* K^+ Channel in the Open State

PUFAs interaction sites in the open and closed state were determined by steered molecular dynamics simulations of *Shaker* K^+ channels in POPC bilayers (Yazdi et al., 2016). PUFAs form clusters around the voltage sensor domain regions (VSD) rather than the pore helices S5–S6. In general, significantly higher contact frequencies were identified between the PUFA and the protein in the open state. PUFA acyl tail-ion channel protein contacts are predominantly non-specific: hydrophobic and distributed across the VSD. PUFA heads-protein interactions are more frequent in the open *vs.* closed state and are more specific: hydrogen bonds between charged or polar residues of the extracellular halves of S3 and S4 and PUFA head groups. Saturated fatty acids established significantly fewer interactions in closed *vs.* open state simulations and fewer contacts in the open state compared to PUFAs. In summary, specific protein residues involved in PUFAs *vs.* saturated fatty acids interactions are largely different. While PUFAs establish predominantly contacts with S3–S4 residues, there is a shift to the S1–S2 in saturated fatty acids (Yazdi et al., 2016).

Place Dependence and Rotation of the First Gating Charge R1 for the PUFA Effect

According to the lipoelectric hypothesis, the PUFA molecule act electrostatically on positive residues of the voltage sensor domain among which arginine 1 (R1) seems to have a crucial role. Shifting the R1 from position 362–361 eliminated the enhancing DHA effect. Moving the charge 2 steps further potentiated the PUFA effect (Ottosson et al., 2014). In the same line of evidence it was found that the introduction of a negative charge (substitution to glutamic acid) in certain positions of the S4 such as A359E, had an opposite effects on the G(V) curve (smaller hyperpolarizing shift compared to WT *Shaker* K^+ channels) to the potentiated effect of arginine (Ottosson et al., 2014). Even more interestingly, the application of AA^+ that was previously shown to decrease WT *Shaker* K^+ current now it up-regulated A359E channels and shifted the G(V) curve −8 mV strongly indicating an electrostatic interaction. AA^+ did not up-regulated A361E showing again amino acid side facing dependence. A positive charge at 359 or a negative at 361 promotes channel opening and vice versa (Ottosson et al., 2014).

According to the structural prediction by Borjesson and colleagues, *Shaker* K^+ channels, with all S4s in an activated but non-conducting position, R1 and R2 are exposed to the extracellular solution with its charges at a distance of 16 Å away from the approximate PUFA position in the lipid bilayer adjacent to S3 and S4 (Borjesson and Elinder, 2011). Assuming that the final position of S4 is according to the $K_V1.2/2.1$ chimera crystal structure (Long et al., 2007), the charge of R1 moves to a position only 6 Å away from the PUFA when the channel is open. A modified Coulomb law was used to estimate the distance between the PUFA carboxyl charge and the S4 mediated change in surface as a function of distance from a charge. The predicted 5 mV effect on the first four independent transition steps suggests that the PUFA charge is located 15.2 Å away from the position where the positive charges emerge on the channel protein's surface. This calculation was based on the assumption that the channel surface is smooth and that the top S4 charges turn up on the surface of the channel leaving the inner part of the ion pore with positive gating charges pairing with negative counter charges. This is very close to the structural prediction of 16 Å. The 30 mV effect on the opening step suggests that the PUFA charge is located 6.3 Å away from R1 in the open state, which again is almost identical to their prediction from the structural model.

Collectively the data strongly suggest that the late transitions (from C2 to O) are the more sensitive steps to DHA. In the last closed state (C4), R1 and R2 are exposed to the extracellular solution 16 Å away from the PUFA head group. In the opening step, R1 moves along the longitudinal axis of S4 and rotate toward the lipid bilayer, in the vicinity of the negatively charged bound PUFA (6 Å; **Figure 2**). DHA electrostatically affects this rotation promoting *Shaker* K^+ channel activation (Borjesson and Elinder, 2011; Ottosson et al., 2014).

Molecular Mechanism of PUFAs-Dependent Modulation of $K_{Ca}1.1$ Channels

Contrary to the DHA effects on *Shaker* K^+ channels, $hK_{Ca}1.1$ channels do not require voltage sensor activation for the PUFA effect. DHA augmented single channel open probability at hyperpolarized potentials where the voltage sensors are predominantly at rest (Hoshi et al., 2013b). In $hK_{Ca}1.1$ channels, opening probability at hyperpolarizing potentials is mainly driven by the opening and closing transitions of the activation gate located near or within the selectivity filter (Seibold et al., 2008). The closure of the activation gate controls the deactivation kinetics and the opening of the gate controls the macroscopic activation kinetics. DHA did not affect the deactivation kinetics of $hK_{Ca}1.1$ channels at hyperpolarized potentials, suggesting that the PUFA accelerates the opening of the activation gate and increases open probability (Hoshi et al., 2013b).

NEW TARGETS FOR PUFAS ION CHANNEL MODULATION

Another crucial aspect needed to determine the beneficial effects of n-3 PUFAs in cardiovascular health is to establish for which pathologies are n-3 PUFAs truly beneficial, neutral or harmful. To do so, it is essential to find new molecular targets responsible for these effects and to analyze how its modulation by PUFAs can affect the physiology in cardiovascular disease models. In this section we summarize recent findings on novel K^+ channels targets involved in vascular relaxation, blood pressure lowering, and prevention of fatal arrhythmias after acute myocardial infarction (AMI).

Potassium Channels As Targets Involved in Vascular Relaxation and Blood Pressure

$K_{Ca}1.1$ Channels

In vascular smooth muscle cells (SMC) human $K_{Ca}1.1$ channels or large conductance Ca^{2+}-activated K^+ channels $hK_{Ca}1.1$ channels together with β1 subunits, have been reported to contribute to the regulation of vascular tone. Activation of $K_{Ca}1.1$ channels acts to keep the membrane hyperpolarized, thus generally exerting a negative feedback influence on cellular excitability (Patterson et al., 2002; Nelson and Bonev, 2004). Consumption of oily fish high in DHA has been suggested to decrease blood pressure in some individuals (Ramel et al., 2010; Saravanan et al., 2010; Liu et al., 2011). Application of DHA dilates isolated blood vessels (Wang et al., 2011) potentially by activating $K_{Ca}1.1$ channels (Lai et al., 2009; Wang et al., 2011). To further examine this hypothesis, Hoshi and colleagues generated a $K_{Ca}1.1$ channel deficient mice model $mK_{Ca}1.1^{-/-}$, and compared the modulatory effect of DHA in aortic vascular SMCs from wild-type (WT) and $mK_{Ca}1.1^{-/-}$mice. Whole-cell outward currents recorded from dissociated aortic vascular SMCs from WT mice were enhanced by extracellular application of DHA but no effect was seen in SMCs from $mK_{Ca}1.1$ deficient mice. Furthermore, the application of DHA into a central vein in WT mice markedly lowered arterial blood pressure. Consistent with the idea that DHA exerts its hypotensive action by activating $K_{Ca}1.1$ channels, injection of DHA into $K_{Ca}1.1^{-/-}$ mice had no effect on blood pressure (Hoshi et al., 2013b). Finally, a bolus of DHA ethyl ester had no effect on blood pressure of WT or $mK_{Ca}1.1$ deficient mice. Together, these results confirmed the blood pressure lowering effect of DHA observed in WT mice is directly mediated by the activation of $mK_{Ca}1.1$ channels in vascular SMCs and highlight $hK_{Ca}1.1$as a major target of the reported PUFA hypotensive effects.

K_{2P} Channels

The two-pore domain potassium channels (K_{2P}) traditionally viewed as "background" (voltage-independent) K^+ channels are dimmers of K_{2P} subunits, each containing four transmembrane segments and two-pore-domain arranged in tandem. K_{2P} channels are mechanosensitive, are active almost instantaneously at all membrane potentials and exhibit strong outward rectification (Lesage and Lazdunski, 2000). K_{2P} channels contribute to the set the resting membrane potential (V_m) in both non-excitable and excitable cells. In excitable cells, K_{2P} channels can contribute to the repolarization as well (MacKenzie et al., 2015). K_{2P} channels are highly expressed in the lung and cerebral vasculature (Gardener et al., 2004; Blondeau et al., 2007) where they have been suggested to participate in the endothelium-dependent vasorelaxation.

A study by Blondeau et al. (2007) showed that alpha linolenic acid induced vasodilation of the basilar artery in WT but not in $K_{2P}2.1^{-/-}$ mice (Blondeau et al., 2007; Garry et al., 2007). More interestingly, it has been suggested that the expression level of K_{2P} channels might be altered in pathological conditions such as pulmonary hypertension (Garry et al., 2007). Pulmonary hypertension is a pathological condition in which there is an increase of blood pressure in pulmonary arteries that can lead hypertrophy of the right ventricle and, finally, heart failure (Hyvelin et al., 2005; Wilkins, 2012). In a comparative study, a 4.4-fold higher density of an endogenous mechanogated K^+ channel with properties resembling those of $K_{2P}2.1$ was found in aortic endothelial cells from spontaneously hypertensive *vs.* normotensive Wistar-Kyoto rats (Hoyer et al., 1997). In the same line of evidence, it was found that $K_{2P}6.1^{-/-}$ knockout mice had a more depolarized V_m in aortic SMC, and a higher systemic blood pressure compared to WT mice (Lloyd et al., 2011). Therefore, it was crucial to determine whether $K_{2P}2.1$ and/or other closely related PUFAs-activated K_{2P} channels would play a crucial role in the prevention and or progression of pulmonary hypertension. In a recent study conducted by Nielsen et al. (2013), the expression of different K_{2P} channels was studied under healthy conditions in the murine lung and in a murine model of pulmonary hypertension induced by hypoxia. In the lung of healthy mice, high expression of mRNA of $K_{2P}2.1$ followed by intermediate levels of $K_{2P}6.1$ and low expression of $K_{2P}4.1$, $K_{2P}10$ were found. Voltage clamp experiments showed PUFAs-sensitive K_{2P} channels in endothelium of the main pulmonary artery and of carotid artery. These currents were blocked by unspecific K_{2P} blockers pimozide and flupentixol. Current clamp experiments in pulmonary artery endothelial cells showed that DHA induced a hyperpolarizing shift of

the V_m of −28 mV. This shift was reversed by pimozide suggesting that DHA caused hyperpolarization through PUFAs-activated K_{2P} channels. DHA activated K_{2P} channels in these cells inducing hyperpolarization and therefore relaxation. Previous studies suggested that vasorelaxation induced by PUFAs in the pulmonary circulation were partially mediated by TEA-sensitive K^+ channels (Morin et al., 2009). Conversely, the study by Nielsen and colleagues showed no attenuation of DHA-induced vasorelaxation when TEA-sensitive channels $K_{Ca}3.1$, $K_{Ca}2.3$, and $K_{Ca}1.1$ channels were inhibited, suggesting that other mechanisms such as K_{2P} activation, play a role in DHA-dependent vasorelaxation. Endothelial denudation did not prevent the DHA effect, indicating that DHA interacts with K_{2P} channels directly in the SMC layers. However, specific K_{2P} inhibitors are required to confirm this assumption. Altered K_{2P} channel expression patterns were observed in hypertensive animals. $K_{2P}6.1$ channels were significantly up-regulated by four-fold in the lungs of chronic hypoxic mice. The physiological meaning of K_{2P} channel upregulation in pulmonary hypertension is not fully understood. Although further experiments are required to clarify this point, this study reveals K_{2P} channels as potential targets in this condition.

Potassium Channel Targets involved in the Prevention of Fatal Arrhythmic Events in the Early Phase of Myocardial Infarction

K_{ATP} Channels

Most deaths due to AMI are caused by ischemia-induced ventricular fibrillation (VF) within the early hours after the onset of infarction (O'Doherty et al., 1983). The GISSI-Prevenzione trial on the effects of long term treatment with n-3 PUFAs demonstrated that these substances reduce sudden cardiac death after AMI (GISSI-Prevenzione Investigators, 1999). K_{ATP} channels have been suggested to provide cardioprotection by promoting action potential shortening and decreasing repolarization dispersion (Chi et al., 1990; Venkatesh et al., 1991). In 2011, Tsuburaya and colleagues conducted the first *in vivo* study to test the potential antiarrhythmic effects of long term EPA treatment on ischemia-induced VF in pigs (Tsuburaya et al., 2011). Myocardial infarction was induced in these animals by left coronary artery occlusion and the electrophysiological outcomes on action potentials were analyzed. The survival rate was 50% in the control group *vs*. 100% in the EPA group. In the control group, many VF episodes caused by triggered extra systoles were recorded during ischemia and terminated by current shocks. Conversely, non-sustained ventricular tachycardia episodes (VT) were recorded in the EPA group. The occurrence of VT/VF and the prevalence of pulseless VT/VF was significantly reduced in the EPA group. After the left coronary occlusion, in the control group, $MAPD_{90}$ (monophasic action potential duration measured at 90% of repolarization) was significantly shortened at the ischemic region while in the EPA group this shortening was significantly attenuated. The pretreatment with cromakalim (K_{ATP} channel opener) abolished the inhibitory effects of EPA on ischemia-induced $MAPD_{90}$ shortening, VT/VF occurrence and deteriorated the survival rate during ischemia in the EPA group to 50%. At the molecular level, EPA treatment significantly reduced cardiac mRNA and protein expression and of $K_{ir}6.2$ (a major component of the sarcolemmal K_{ATP} channel) and increased that of the auxiliary SUR2B subunit. Taken together, these findings indicate that EPA suppressed acute phase fatal ventricular arrhythmias (15 min after onset). EPA had an inhibitory effect on ischemia-induced VF *in vivo* probably mediated by amelioration of ischemia-induced monophasic action potentials shortening (Tsuburaya et al., 2011). Nevertheless, patch clamp studies are needed to demonstrate inhibition of K_{ATP} in cardiomyocytes.

NEW DISCOVERIES IN K_V7 FAMILY

The $K_V7.1$ family of voltage-gated K+ channels comprises $K_V7.1$–$K_V7.5$ channels. In the heart, $K_V7.1$ channels co-assemble with KCNE1 subunits to give rise to the slowly activating delayed-rectifier K^+ current I_{Ks}. This current contributes importantly to cardiac repolarization particularly during β-AR stimulation (Sanguinetti et al., 1996; Volders et al., 2003). The other four components of the K_V7 family ($K_V7.2$–$K_V7.5$) are mainly expressed and studied in the nervous system, although they play important roles in other tissues as well (i.e., vascular smooth cells, ear cells, etc.). $K_V7.2$ and $K_V7.3$ are the major components of the slow voltage-activated M-current. $K_V7.2/K_V7.3$ channels are opened already at rest hence contributing to the V_m of dorsal root ganglion (DRG) and hippocampal neurons, and regulating neuronal excitability (Jentsch, 2000; Brown and Passmore, 2009).

PUFAs and Cardiac I_{Ks} ($K_V7.1$/KCNE1)

As with many other K^+ channels, $K_V7.1$ channels and, therefore, I_{Ks} are modulated by PUFAs. Doolan et al. (2002) demonstrated that EPA increases the magnitude of the I_{Ks} in *Xenopus* oocytes. Furthermore, long term PUFA treatment was shown to enhance I_{Ks} in cardiac myocytes from pigs pigs fed on a PUFA high diet compared to I_{Ks} from pigs fed with a normal diet (Verkerk et al., 2006). However, the molecular mechanism underlying the PUFAs-dependent modulation of $K_V7.1$/KCNE1 remained to be elucidated. In 2015, two studies addressed this question (Liin et al., 2015; Moreno et al., 2015).

Moreno et al. (2015) analyzed the effects EPA and DHA in $K_V7.1$/KCNE1 channels expressed on a heterologous expression system and in I_{Ks} in guinea pig myocytes. Two aspects of PUFA-dependent modulation of ion channels merit particular attention. (i) Dietary PUFAs incorporate to the cell membrane. Therefore, PUFAs-dependent modulation of ion channel depend on the way of administration: acute *vs.* chronic [see Moreno et al. (2012) for extensive review]. In fact, Moreno and colleagues reported that acute but not chronic administration of PUFAs increased the magnitude of $K_V7.1$/KCNE1 channels and shifted the activation curve toward opposite directions (leftward and rightward shift for acute and chronic administration, respectively). In addition, long-term administration of n-3 PUFAs reduced $K_V7.1$ but not KCNE1 expression due to increased degradation via proteasome. The significant decrease in $K_V7.1$ expression level might account for the lack of current density increase when n-3 PUFAs were

applied chronically (Moreno et al., 2015). (ii) Direct *vs.* indirect effects. As previously mentioned, there were two trends of opinion to explain the mechanism of action by which PUFAs modify ion channels: direct interaction *vs.* alteration of the cell membrane fluidity. Evidences in favor of both type of modulation were found simultaneously in the study by Moreno et al. (2015). Chronic administration of EPA and DHA disrupted lipid rafts domains where $K_V7.1$ channels are normally found (Balijepalli et al., 2007) delocalizing $K_V7.1$ in the cell membrane. Lipid raft disruption can be mimicked within minutes by the acute application of methyl-β-cyclodextrin (MBCD). The application of MBCD increased the current magnitude of $K_V7.1$/KCNE1 at potentials positive to +30 mV and shifted the activation curve toward positive potentials emphasizing the impact of the membrane environment on channel properties (indirect effect; **Figure 4**; Moreno et al., 2015). On the other hand, after lipid raft disruption with MBCD, acute application of EPA produced similar effects than those observed in non-cholesterol-depleted cells: Current density increase and shift of the midpoint in the hyperpolarizing direction. This fast modulation was explained by a direct effect on the ion channel of EPA during acute application. Together these data provided evidence for direct and indirect PUFA dependent modulation of K^+ channels suggesting that both mechanisms are not mutually exclusive and can happen simultaneously (Moreno et al., 2015).

The study by Liin et al. (2015) examines the mechanism by which n-3 PUFAs promote $K_V7.1$ channel opening and how KCNE1 modulated $K_V7.1$ sensitivity to PUFAs. In addition, they explored the possibility to develop PUFAs analogs as drugs to prevent cardiac arrhythmias. Similarly to what was described for *Shaker* K^+ channels, DHA promotes channel opening by acting electrostatically on the VSD of $K_V7.1$ channels. KCNE1 tunes $K_V7.1$ sensitivity to PUFAs by lowering the local pH in the vicinity of the PUFA head group which results in DHA protonation. In other words, the PUFA effect is KCNE1-independent as long as the PUFA is negatively charged (Liin et al., 2015). This finding raised the possibility to study the antiarrhythmic potential of n-3 PUFAs analogs as novel therapeutic agents. In this regard, Liin and colleagues tested the effects of two PUFAs analogs in two arrhythmia models: isolated embryonic rat cardiomyocytes and the intact heart of guinea pig. In isolated embryonic rat cardiomyocytes (in which I_{Ks} but not I_{Kr} rapidly activating delayed rectifier potassium current is expressed) arrhythmia was induced by applying subsaturating concentrations of chromanol 293B and the effects of N-arachidonoyl taurine were tested. Chromanol 293B increased action potential duration and led to arrhythmic firing. The permanently negatively charged N-arachidonoyl-taurine reversed the effect of chromanol 293B decreasing the action potential duration (APD) and abolishing the arrhythmic

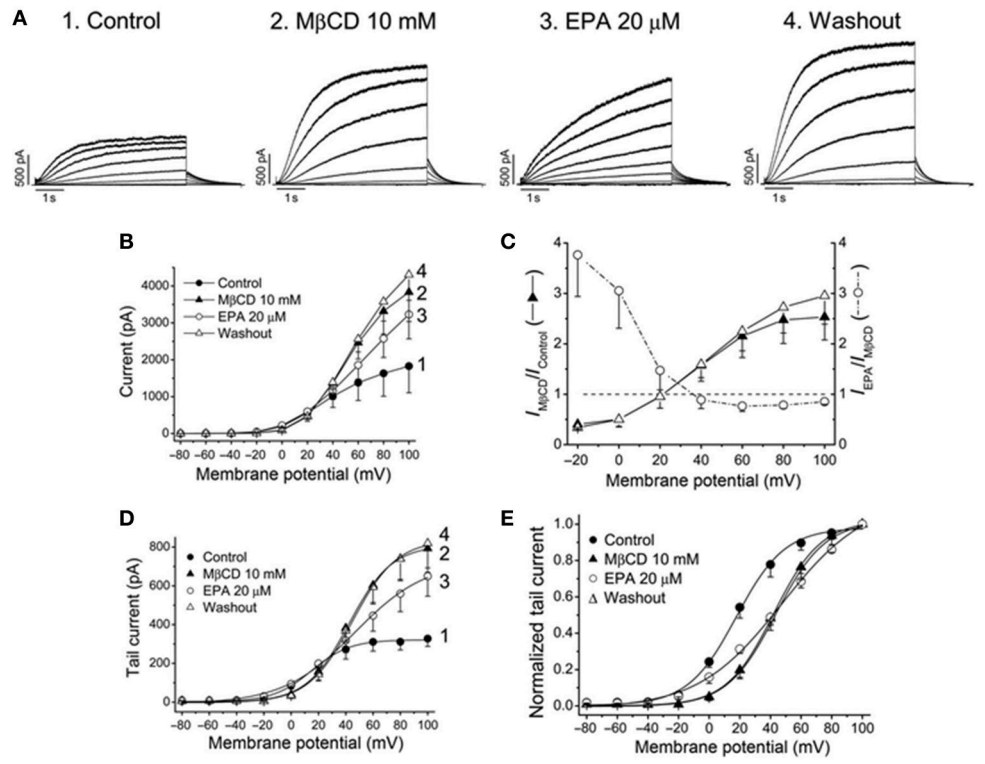

FIGURE 4 | Effects of EPA in membrane cholesterol-depleted cells with methyl-β-cyclodextrin (MβCD). (A) Current records obtained from the same cell and generated by $K_V7.1$-KCNE1 channels in control, after perfusion with MβCD, with EPA and after washout the cells with EPA-free external solution. **(B)** Current-voltage relationships under all conditions shown in **(A)**. **(C)** Plot showing the voltage dependence of the MβCD and EPA effects **(D)** Activation curves and **(E)** normalized activation curves. Moreno et al. (2015).

firing. The effects of docosahexaenoyl glycine were tested on the intact heart of guinea pig (DHA-Gly has a lower pKa than DHA and therefore is negatively charged at physiological pH even in the presence of KCNE1). To mimic the long QT (syndrome characterized by a long interval between the Q and the T waves of the ECG) setting in this model (APD duration and QT prolongation), the E4031 I_{Kr} blocker was applied. Perfusion of the guinea pig heart with 10 μM DHA-Gly reversed the effects of E4031 restoring QT interval duration (Liin et al., 2015) demonstrating their antiarrhythmic potential.

Concerning the PUFAs antiarrhythmic mechanism, minor discrepancies can be found through the literature. Overall, *in vitro* and *in vivo* experiments and clinical trials support an antiarrhythmic effect of PUFAs. Liin and colleagues claim that the antiarrhythmic effects of DHA cannot be due to effects on I_{Ks} gating because at physiological pH 7.4, KCNE promotes DHA protonation making DHA ineffective (Liin et al., 2015). Instead they propose that the antiarrhythmic effect of PUFAs in the heart is due to the inhibition of I_{Na} or I_{CaL} (Leaf et al., 2003, 2005; Danthi et al., 2005; Boland and Drzewiecki, 2008). However, we found that both EPA and DHA increase I_{Ks} in guinea pig cardiomyocytes and shifted the G(V) curve toward the hyperpolarizing direction of I_{Ks} expressed in COS7 cells. Furthermore, action potential simulations showed that the increase of I_{Ks} induced by acute administration of PUFAs could play a relevant role in action potential shortening when other repolarizing current for instance I_{Kr}, are compromised. On the other hand, chronic exposure to PUFAs does not affect APD so that the protective role seems lost in this condition.

PUFAs and Neuronal M-Current

It has been reported that PUFAs increase both seizure and pain thresholds (Voskuyl et al., 1998; Xiao and Li, 1999; Taha et al., 2010; Bandero et al., 2013) in rats and mice, and reduce neuronal excitability *in vitro* (Xiao and Li, 1999; Young et al., 2000). As it was shown for the heart, the modulation of neuronal excitability by PUFAs is mediated by effects on ion channels crucial for neuronal excitability (Boland and Drzewiecki, 2008). PUFAs have been suggested to increase the M-current but their effects are complex and not completely understood. In a recent paper, it was shown again that only negatively charged n-3 PUFAs such as DHA, EPA, ALA, can quickly shift the G(V) curve of $K_V7.2/K_V7.3$ channels expressed on *Xenopus* oocytes toward more negative voltages facilitating channel opening (Liin et al., 2016; Valenzuela, 2016). More importantly, the anti-excitable effects of PUFAs on the neuronal M-current were studied in dorsal root ganglions from mice. Addition of DHA to dorsal root ganglion neurons hyperpolarized the V_m (by −2.4 mV) increasing the threshold current required to evoke action potentials. Since these effects were reverted by the M-current blocker XE991, the reduced neuronal excitability was attributed to the PUFA mediated augmentation of M-current (Liin et al., 2016). In the same line of evidence, computer simulations predict that equivalent changes around 1–5 mV in the V_m of neurons and in the voltage-dependence of ion channel activation is sufficient to decrease neuronal excitability and prevent epileptic episodes (Tigerholm et al., 2012). In summary, all these results suggest that PUFAs can be used as a blueprint for rational drug design of new M-currents activators more selective and efficient for the treatment of neurological disorders such as epilepsy and pain.

CONCLUDING REMARKS

From 1990's we know that PUFAs produce diverse effects on ion channels that might be beneficial for health. The mechanism of action initially proposed by the Elinder's group: The lipoelectric hypothesis (the lipid bilayer or bilayer/channel interface close to the S3–S4 segment) from where they act electrostatically on the voltage sensor and thereby change the voltage dependence of the channel. The modulation of potassium channels by PUFAs is channel specific, as indicated by the identification of two PUFAs binding sites in different potassium channels families. Besides the gating modulation of ion channels different studies have demonstrated that PUFAs can modulate differentially ion channels for instance through the modulation of the biophysics of the lipid bilayer, by targeting the channels to different areas of the cell membrane (i.e., lipid rafts) and even modifying the gene expression. Finally, other ion channels such as $K_{Ca}1.1$, K_{ATP}, and K_{2P} have been recently reported as sensitive to the effects of PUFAs. Since these potassium channels are present in the cardiovascular and in the nervous systems, PUFAs might play an important role in the control of blood pressure, cardiac arrhythmias, and neurological disorders.

AUTHOR CONTRIBUTIONS

CM, AC, and CV wrote this review.

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REVIEW
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Modulation of the Activities of Neuronal Ion Channels by Fatty Acid-Derived Pro-Resolvents

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Progress of inflammation depends on the balance between two biological mechanisms: pro-inflammatory and pro-resolving processes. Many extracellular and intracellular molecular components including cytokines, growth factors, steroids, neurotransmitters, and lipidergic mediators and their receptors contribute to the two processes, generated from cellular participants during inflammation. Fatty acid-derived mediators are crucial in directing the inflammatory phase and orchestrating heterogeneous reactions of participants such as inflamed cells, innate immune cells, vascular components, innervating neurons, etc. As well as activating specific types of receptor molecules, lipidergic mediators can actively control the functions of various ion channels via direct binding and/or signal transduction, thereby altering cellular functions. Lipid mediators can be divided into two classes based on which of the two processes they promote: pro-inflammatory, which includes prostaglandins and leukotrienes, and pro-resolving, which includes lipoxins, resolvins, and maresins. The research on the modulations of neuronal ion channels regarding the actions of the pro-inflammatory class has begun relatively earlier while the focus is currently expanding to cover the ion channel interaction with pro-resolvents. As a result, knowledge of inhibitory mechanisms by the pro-resolvents, historically seldom found for other known endogenous modulators or pro-inflammatory mediators, is accumulating particularly upon sensory neuronal cation channels. Diverse mechanistic explanations at molecular levels are being proposed and refined. Here we overviewed the interactions of lipidergic pro-resolvents with neuronal ion channels and outcomes from the interactions, focusing on transient receptor potential (TRP) ion channels. We also discuss unanswered hypotheses and perspectives regarding their interactions.

Keywords: resolvin, neuroprotectin, maresin, ion channel, neuron, modulation

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INTRODUCTION

Inflammation is a protective mechanism of the body against physical/chemical injury, or infection. The inflammatory reaction is associated with functional changes in diverse tissues and cell types. In the past, the progression of inflammation from its initiation through termination was believed to be dependent only on pro-inflammatory mechanisms on the molecular and cellular levels. That is, when pro-inflammatory mediators are upregulated, the inflammatory response may last or even

deteriorate because of activations of the cellular players and their signal transduction. Conversely, when those are downregulated, the inflammatory response may passively subside. This indicates that pro-inflammatory mediators, secreted in response to insults, stimulate the participant cells and tissues to become or remain inflamed. In the absence of such stimulation, however, the cells and tissues passively recover from the inflamed state. In the early 2000's, this hypothesis began to be revised (Serhan, 2002). Resolution became to be viewed as an active, rather than a passive, process that is accelerated by endogenous pro-resolving agents. Accordingly, when the aim is to fight with pathologic inflammation, the promotion of pro-resolving processes can be hypothesized as an important goal as well as the suppression of the pro-inflammatory processes. To understand the details of this resolution paradigm on the cellular and molecular levels, the identities and functions of the pro-resolving molecules (hereafter referred to as pro-resolvents) have been intensely studied in recent years (Serhan et al., 2015). Among heterogeneous pro-resolving substances including peptides, nucleosides, steroids, and neurotransmitters, here we focus on fatty acid-derived ones. To date, about 30 pro-resolvents have been found in endogenous fatty acid pools in inflamed exudates. Most of these are metabolites of n−3 fatty acids, including E-series resolvins (RvEs), D-series resolvins (RvDs), maresins (MaRs), and neuroprotectin (NPD1).

Changes in cellular functions upon exposure to pro-resolvents are an important aspect of the resolution paradigm. A critical parameter driving these changes is ionic transport. Fluctuations in ion (e.g., Ca^{2+}) concentrations can significantly affect intracellular signal transduction in many cell types. Moreover, ionic flux is essential for controlling neuronal excitability. Therefore, pro-resolvent interactions with cellular ion-permeating channels, if any, might be an important node for altering the inflammatory phase of cells. In fact, two axial mechanisms regarding such interactions have emerged. One involves ion channel modulation related to sensory neuronal excitability and the other involves that in mucus secreting pathways in the airway (Karp et al., 2004; Ji et al., 2011; Yoo et al., 2013; Lim et al., 2015). The molecular details of these two mechanisms have been investigated in the context of inflammatory pain sensation and cystic fibrosis, respectively. These studies have also suggested potential outcomes from the interactions between pro-resolvents and ion channels. Of these two mechanisms, we focus on the modulation of sensory neuronal ion channels and discuss further derived hypotheses for related disease progress and future perspectives.

FATTY ACID-DERIVED PRO-RESOLVENTS

Fatty acid metabolites are produced from immune cells including monocytes/macrophages, injured tissue, and the vascular endothelium during inflammation. After extracellular diffusion, these substances can be further processed biosynthetically to become final regulatory mediators which in turn interact with their partner receptor molecules. These interactions drive alterations in cellular function. These lipidergic components can be divided into two categories according to their regulatory directions in the inflammatory phases: pro-inflammatory and pro-resolving classes (Serhan et al., 2008; Ji et al., 2011). Although, a few exceptions exist, substances derived from n−6 fatty acids (e.g., prostaglandins and leukotrienes) generally serve pro-inflammatory roles. By contrast, pro-resolvents predominantly include n−3 derivatives.

Notable exceptions are lipoxin species, which are metabolites of n−6 arachidonic acid that exhibit pro-resolving activity. Among the lipidergic pro-resolvents, lipoxins were the first to have their chemical structures characterized and their biological actions identified (Serhan et al., 1984). Lipoxin was also the first lipidergic pro-resolvent to be studied in terms of an ion channel-associated action. In 1992, around which the curiosity regarding lipid-ion channel interaction was increasing due to the findings that some eicosanoids were shown to activate K^+ channels (Buttner et al., 1989; Kim and Clapham, 1989), lipoxin A4 was demonstrated to excite dorsal root ganglionic sensory neurons as measured by surrogate outputs of increased bronchus contraction and neuropeptide secretion (Meini et al., 1992). Blockage and desensitization of the vanilloid subtype transient receptor ion channel (TRPV1) each prevented this effect, although it was not electrophysiologically confirmed, implicating that TRPV1 activity may be enhanced by lipoxin A4 exposure. Lipoxin A4 did not exhibit competitive binding with a TRPV1 agonist to the natural protein in the neuronal membrane. However, early binding assays were not perfectly reliable as it was later shown that TRPV1 protein was not actually used in this assay (Meini et al., 1992). Thus it remains elusive whether lipoxin A4 directly binds to this ion channel or indirectly modulates its activity via signal transduction events to exert this excitatory effect. It was considered unlikely that this n−6 derived lipoxin A4 resulted in neuronal excitation, which possibly conflicts with other known resolution mechanisms by which excitatory features are believed to be down-regulated. In contrast, n−3 metabolites, which constitute the majority of pro-resolvents, have commonly been shown to suppress TRP channel activities, eventually interfering with neuronal excitation, as described below.

Although, this review will not focus on this topic, the effects of lipoxins on ionic conductance have been studied more often in the context of airway fluid balance than in neuronal excitability (Karp et al., 2004). Lipoxins have been shown to promote the activity and/or expression of many different types of ion channels including epithelial Na^+ channels, cystic fibrosis transmembrane conductance regulators, ATP-sensitive K^+ channels, Ca^{2+}-activated anion channels, pannexin 1 hemichannels, and canonical subtype of TRP channels (TRPCs). These effects lead to beneficial outcomes with respect to fluid secretion in the airway (Pang et al., 2011; Verriere et al., 2012; Buchanan et al., 2013; Wang et al., 2013; Yang et al., 2013; Al-Alawi et al., 2014; Higgins et al., 2014, 2015; Qi et al., 2015). Recent studies showing similarly beneficial effects from treatment of other n−3 type pro-resolvents have also emerged in the airway field (Hiram et al., 2014; Wang et al., 2014; Colby et al., 2016).

More than a decade after the discovery of lipoxins, n−3 type lipids began to be added in the list of pro-resolvents (Serhan

et al., 2000). The n−3 fatty acid-derived pro-resolvents include D-series and E-series resolvins, their isomers, neuroprotectin D1, and maresins (Yoo et al., 2013). Variant intermediates are first produced from source lipids [e.g., n−3 docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA)] via pre-oxygenating processes by cytochrome P450, aspirin-triggered cyclooxygenase, or 12- or 15- lipoxygenases (LOXs). Next, 5-LOX generates the final forms of all these pro-resolvents. Each enzymatic step can occur in different types of participant cells including monocytes/macrophages, epithelial cells, and platelets, through intercellular diffusions of the intermediates, which is called transcellular biosynthesis (Yoo et al., 2013). Although, this process likely starts at the same time as the production of pro-inflammatory mediators such as various prostaglandins, the tissue levels of pro-resolvents seem to peak hours to days later, which may reflect the time needed for *de novo* transcription of the enzymes that produce pro-resolvents. Therefore, this time lag contributes to the duration of the inflammatory response.

The activation of specific G-protein coupled receptors (GPRs) and the resultant changes in cellular function explain the molecular mechanism for many of the actions of pro-resolvents on innate immune cells and inflamed tissues. For example, RvE1 (E designates the precursor lipid, EPA) activates the GPR, chemR23, and antagonizes the type 1 leukotriene B4 receptor (BLT1). RvD1 (D designates DHA) activates GPR32 and lipoxin A4/Annexin-A1 receptor/formyl-peptide receptor 2 (ALX/FPR2). RvD5 has also been shown to activate GPR32. RvD2 activates GPR18 (Lim et al., 2015). It is unclear whether other pro-resolvents also utilize GPR signaling. The cellular outcomes of these molecular actions are variable but commonly contribute to resolution: e.g., decreased production and secretion of pro-inflammatory mediators from immune cells and tissues, disturbance of recruitment and function of the polymorphonuclear neutrophils, increased recruitment of monocytes/macrophage; enhanced non-phlogistic phagocytosis of innate immune cells, and increased secretion of peptidergic pro-resolvents such as interleukin-10 from those cells (Yoo et al., 2013).

After accumulation of the efficacy indices and mechanistic information about the actions of pro-resolvents in the immunology field, scientific interest extended to include the relation with neuronal ion channels. This interest took 10 years to develop, longer than the period that it took from lipoxin finding to its historical approach targeting TRPV1 (Xu et al., 2010). It may be partly attributed to the limited availability of substances for the academic field to study. Most of the results have been produced from six pro-resolvents and four sensory neuronal TRP ion channels, which will be introduced, following a brief overview of the tradition of TRP channel-lipid interaction studies.

TRP-LIPID INTERACTIONS

Mammals, including humans, express 28 or 29 TRP ion channel subtypes. Different from other six-transmembrane channels, most of which serve as voltage-gated channels, TRPs are only weakly sensitive to changes in the membrane voltage and play heterogeneous roles. In response to stimuli specific for each TRP, e.g., physical stimuli such as temperatures and mechanical forces, binding of second messengers, enzymatic modifications of the protein, and endogenous chemical activators, TRP channels open and allow predominantly Ca^{2+} and Na^{+} to permeate. This process leads to the depolarization of excitable cells or to the promotion of various Ca^{2+}-mediated signaling processes in both excitable and non-excitable cells. With respect to the chemical binding, few cases of inhibitory modulation of TRP activity have been found but activation and potentiation constitute majority of reactions (Yoo et al., 2014).

Excluding the lipoxin experiment with native TRPV1 mentioned above owing to the lack of direct measurement of channel activity, the tradition of lipid interaction studies in the TRP field began in the late 1990s (Chyb et al., 1999; Zygmunt et al., 1999; Hwang et al., 2000). As soon as n−6 polyunsaturated fatty acids were reported to activate *Drosophila* TRP channels (Chyb et al., 1999), anandamide, hydroperoxyeicosatetraenoic acids (HPETEs), and leukotrienes, all of which are also n−6 fatty acid derivatives, were identified as TRPV1 activators (Zygmunt et al., 1999; Hwang et al., 2000) Since then, information on lipid activators has been expanded dramatically (Choi et al., 2014; Lim et al., 2015). The activities of TRPC and melastatin type TRP (TRPM) species are affected by the binding of second messengers such as diacylglycerol or its precursor, phosphatidylinositol 4,5-bisphosphate. Ankyrin type 1 TRP (TRPA1) is highly responsive to a broad range of lipid peroxides via a unique binding mode whereby peroxygenated lipids covalently bind to its cytosolic N-terminal nucleophilic amino acid residues. TRPVs tend to be activated by hydroperoxy- or epoxy-eicosanoids. Recent structural approaches have identified a putative lipid binding site in the intracellular regions of TRPV1 protein as described below. Most of those lipids can activate only their specific TRP channel and can also synergistically potentiate its activity in the presence of a stimulus with a different quality. These lipid activators are generated by various cells, even cells expressing TRPs. These molecules appear to behave in an autocrine and paracrine manner. TRP activations by these lipids in neurons mostly promote their excitability.

MODULATION OF SENSORY TRP ACTIVITIES BY PRO-RESOLVENTS

Several TRPs serve as molecular sensors for environmental changes, expressed particularly highly in the skin-innervating terminals of somatosensory nerve C-fibers and Aδ fibers. Among 28 human subtypes, 6 members, which are TRPA1, TRPV1-4, and TRPM8, play this role and are collectively called sensory TRPs. These sensory TRP channels are all polymodal to some extent, which might be an evolutional strategy for enabling sensory neurons to monitor a variety of environmental changes. All 6 of these TRPs are responsive to a particular range of temperatures and 3 of them are sensitive to different qualities of mechanical stretching (Hwang and Oh, 2007). Regarding chemical sensitivities, a surprisingly large number of natural

pungent substances, toxins, and different kinds of endogenous lipids, have been shown to activate sensory TRPs. In specialized sensory organs in the vision, olfaction, and gestation systems, the majority of sensor molecules are specific types of GPRs and TRP channels predominantly function as downstream effectors driving depolarization. In contrast, sensory TRPs seem to be the major receptors to detect environmental risks in the somatosensory system. This setup likely benefits from the high degree of polymodality of TRPs. Rather than with pleasant or explorative quality, perception by our brain resulting from TRP activation is with a nociceptive one: pain. This attribute frequently places TRP modulation as a desirable goal at the viewpoint of analgesia which can be rephrased as resolution of pain (Lim et al., 2015). Many studies of the interactions of TRPs with fatty acid-derived pro-resolvents have adhered to this angle. Six pro-resolvents have been investigated in terms of their actions on five different neuronal ion channels, four of which are TRPs. All the pro-resolvents negatively modulated the ion channel activities, indicating that these lipids are potential analgesics (**Table 1**).

TRPA1 Modulations by D-Series Resolvins and MaR1

As detailed above, the TRPA1 ion channel is expressed in only a subpopulation of pain-mediating nociceptors. Some types of physical and chemical insults that are potentially harmful to the body can affect the structure of TRPA1 protein, leading to allosteric opening of this channel. Thus TRPA1 is often called a "damage-sensing ion channel." TRPA1 is particularly sensitive to noxiously cold temperatures (<17°C; Story et al., 2003; Obata et al., 2005; Kwan et al., 2006; Karashima et al., 2009; Del Camino et al., 2010; Chen et al., 2011), harsh mechanical deformations (Petrus et al., 2007; Kerstein et al., 2009), and the binding of environmental or endogenously generated toxic substances including diverse lipid peroxides, reactive chemicals, and pollutants (Bang and Hwang, 2009; Kim and Hwang, 2013). In response to these stimuli, TRPA1 opens and depolarizes the nociceptors. Hardly shown in activation mechanisms for other ion channels, covalent reactions with specific nucleophilic amino acids in the cytosolic N-terminus of TRPA1 with α,β-unsaturated carbon-containing substances seem to underlie the ligand-mediated gating of TRPA1 (Hinman et al., 2006; Macpherson et al., 2007; Andersson et al., 2008; Bang and Hwang, 2009). In addition, TRPA1 also acts as a final effector in intracellular signal transduction for inflammatory mediators such as nerve growth factor (NGF) and bradykinin, indicating that TRPA1 is critical for inflammatory pain sensation (Bandell et al., 2004; Bautista et al., 2006; Kwan et al., 2006; Petrus et al., 2007; Cenac, 2013). Due to this polymodality and the distinctively limited number of TRPA1-positive neurons, these neurons are frequently subcategorized into polymodal nociceptors. The identification of a mechanism for inhibiting TRPA1 activity, would greatly contribute to the resolution of pain states (Baraldi et al., 2010).

Of particular note, pro-resolvents seem to have a central role for endogenous inhibition of TRPA1. For instance, resolvin D1 (RvD1) strongly blocks TRPA1 activity (Bang et al., 2010). This channel blocking potency indeed extrapolates to the *in vivo* level by showing that TRPA1 agonist-induced chemical pain were attenuated by RvD1 administration. TRPA1 also takes part in the mechanosensory mechanisms, and RvD1 was shown to prevent mechanical hypersenstivity in complete Freund's adjuvant (CFA)-inflamed animals. Since RvD1 is locally confined when injected and outcomes were measured at relatively acute time points, the pain relief observed *in vivo* appears to be mediated in the absence of intercellular actions such as recruitment or activation of innate immune cells.

RvD2 also inhibits TRPA1 activity (Park et al., 2011b). G-protein-mediated signal transduction seems to intervene the molecular mechanism of this process while such an explanation is unlikely for RvD1: the inhibitory action of RvD2 was shown to be prevented by the uncouplers for Gαi-cycling, pertussis toxin, and GDPβS. The specific GPR that initiates the signal has yet to be identified. On the other hand, specific agonists for ALX/FPR2, the GPR on which RvD1 acts, such as cathelicidin LL-37 and Trp-Lys-Tyr-Met-Val-Met (WKYMVM) are inert to TRPA1 activation or inhibition (Bang et al., 2010). To confirm their mechanism of action, the different modes for these related substances for the same target protein may need to be revisited (**Figure 1**). Subtle structural difference (e.g., differences in the location of one hydroxyl moiety and in the cis-trans nature of carbon links) may determine the receptor binding specificity. With respect to behavioral outcomes, no significant discrepancy was observed. TRPA1-mediated mechanical phenotype was greatly improved under inflamed conditions (Park et al., 2011b).

MaR1 is a recently identified pro-resolvent that has been demonstrated to elicit somewhat contradictory outcomes. *In vitro* electrophysiological measurements indicated that TRPA1 activity is not affected by the presence of MaR1 (Serhan et al., 2012). However, vincristine-induced *in vivo* mechanical hypersensitivity, to which TRPA1 is known to contribute, was significantly alleviated by MaR1 treatment. Without direct TRPA1 involvement, MaR1 may act

TABLE 1 | List of neuronal ion channels that are modulated by lipidergic pro-resolvents.

Ion channel	Lipidergic pro-resolvents
TRPA1	Resolvin D1 (Bang et al., 2010), Resolvin D2 (Park et al., 2011b), Maresin 1 (Serhan et al., 2012)
TRPV1	Resolvin D2 (Park et al., 2011b), Resolvin E1 (Xu et al., 2010; Park et al., 2011b), Neuroprotectin D1 (Park et al., 2011a), Maresin 1 (Serhan et al., 2012; Park, 2015)
TRPV3	AT-resolvin D1 (Bang et al., 2012a), Resolvin D1 (Bang et al., 2010)
TRPV4	Resolvin D1 (Bang et al., 2010)
NMDA receptor	Resolvin E1 (Xu et al., 2010), Resolvin D1 (Quan-Xin et al., 2012)

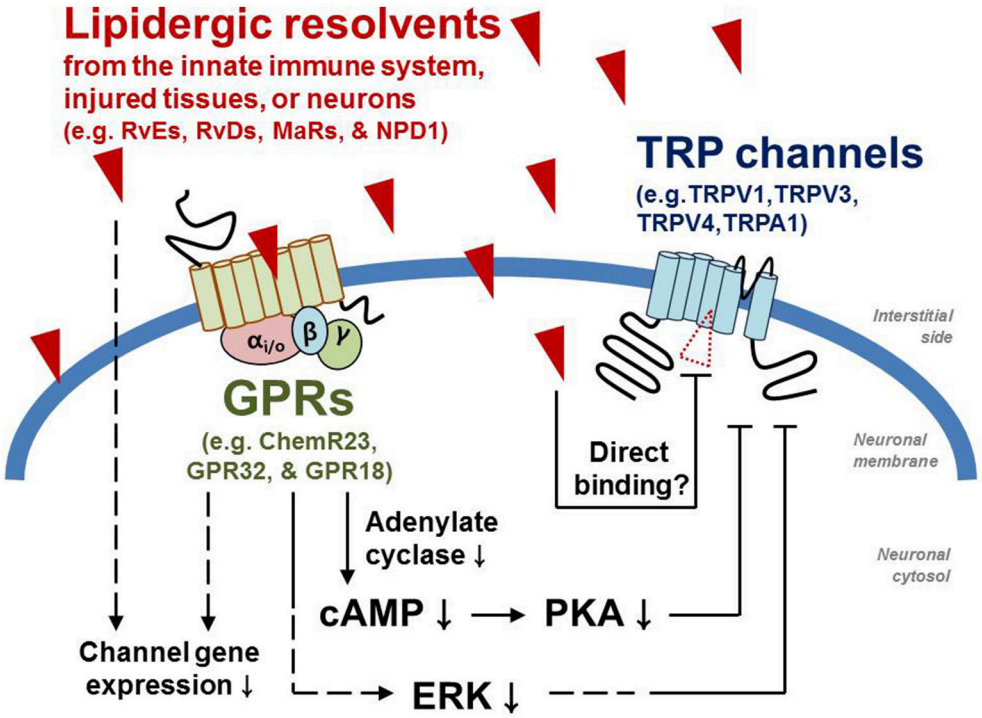

FIGURE 1 | Molecular modes of action by which pro-resolvents inhibit TRP channels. Dotted lines indicate that other unknown signaling components may take part during the pathways.

downstream or upstream of TRPA1 mediation in pathological processes.

TRPV1: An Important Node for Various Resolving Actions

With a greater polymodality than that of TRPA1, TRPV1 serves as a central pain receptor in the body. Potentially damaging heat (>42°C), acidosis, abnormal hypertonicity, endogenous lipidergic pro-inflammatory messengers such as leukotrienes, and natural pungent compounds such as capsaicin and tarantula toxin evoke pain via direct structural challenges to TRPV1 protein, culminating in its electrical activation (Caterina et al., 1997, 2000; Tominaga et al., 1998; Davis et al., 2000; Hwang et al., 2000; Siemens et al., 2006; Vriens et al., 2009; Nishihara et al., 2011). Furthermore, major pro-inflammatory mediators such as bradykinin, prostaglandin, and NGF utilize TRPV1 as their downstream effector, thereby causing pathologic exacerbation of pain and inflammation (Basbaum et al., 2009). These findings have implicated TRPV1 as the forefront node between painful insult and human and mammalian perception of$ it (Hwang and Oh, 2007). While such nodal actions are well-defined in the periphery of the sensory neurons, evidence is currently expanding for its contribution in the central terminals and even in spinal interneurons to the boosting pain circuit, which suggests that its different nodal roles exist in the central synapses (Choi et al., 2016). Therefore, TRPV1 may also be a critical pain resolving node because the extensive scale of pain pathologies could be controlled if TRPV1 activity could be tuned.

In the same way for TRPA1 inhibition, RvD2 suppresses TRPV1 activity. Through unknown GPR activation by RvD2, intracellular Gαi-coupled signaling leads to decreased TRPV1 activity. Of all the pro-resolvents whose ability to inhibit TRP has been studied, RvD2 exhibits the strongest potency (IC50 ~100 pM). Predictably, acute pain induced by capsaicin and inflammatory heat hyperalgesia, which are the *in vivo* modalities that TRPV1 predominantly covers, were both blunted by RvD2 treatment (Park et al., 2011b).

Similarly, RvE1 and MaR1 have been found to inhibit TRPV1 activity and TRPV1-mediated pain behavior, but did not affect modalities involving TRPA1 (Xu et al., 2010; Park et al., 2011b; Serhan et al., 2012; Park, 2015). Since RvE1 acts on a single GPR (chemR23), it can be hypothesized that certain GPR signaling pathways may be more tightly coupled to specific TRP subtypes. In fact, coexpression of chemR23 and TRPV1 has been confirmed in a subset of nociceptors (Xu et al., 2010).

Neuroprotectin D1 (also called protectin D1 or NPD1), another D-series pro-resolving lipid, has also been shown to inhibit TRPV1 albeit one third of the potency of RvD2 (Park et al., 2011a). A Gαi protein-coupled indirect mechanism was again suggested to underlie this inhibitory action, although the specific GPR involved is not known. Other signaling components that can amplify TRPV1activity may also be affected by NPD1, for example, protein kinase A (PKA) and extracellular signal–regulated kinase (ERK; Park et al., 2011a). Moreover,

TNF-α-mediated spinal synaptic transmission was attenuated and the associated behavioral phenotypes were extrapolated in a manner partly dependent on TRPV1 inhibition. Recently, NPD1 treatment also exhibited beneficial effects on neuropathic pain in an animal study (Xu et al., 2013).

TRPV3 Modulation by D-Series Resolvins

Several aspects may make one hesitate to conclude that TRPV3 is a nociceptive component. For example, TRPV3 begins to open in response to relatively mild heat radiation that results in innocuously warm temperatures (>33°C), whereas the temperature threshold for TRPV1 activation is close to the range reported to actually cause pain perception *in vivo* (Caterina et al., 2000; Moqrich et al., 2005). The main expresser cells are not sensory neurons but non-excitable basal keratinocytes in the epidermis (Peier et al., 2002). The presence of TRPV1 seems to be sufficient to monitor external noxious temperatures (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002, 2006). Although, the precise intercellular circuitry involved in TRPV3-mediated somatosensory signaling has yet to be fully unraveled, pharmacological, and genetic studies have yielded some insights. Industrial drug developments targeting TRPV3 have shown that synthetic TRPV3 blockers commonly exhibit painkilling efficacy in inflammatory and neuropathic pain (Facer et al., 2007; Bevan et al., 2008; Broad et al., 2009; Khairatkar Joshi et al., 2010; Reilly and Kym, 2011). More importantly, TRPV3-knockout animals have shown significant tolerance to painful heat (Moqrich et al., 2005; Huang et al., 2008; Miyamoto et al., 2011). Thus, TRPV3 participates in nociception of noxiously high temperatures albeit to a lesser extent than TRPV1, and keratinocytes may contribute to thermosensation possibly by transmitting their depolarization signals to neighboring nociceptor neurons.

Consistently, both resolvins that modulate TRPV3 activity exert painkilling effects. Aspirin-triggered RvD1 (AT-RvD1 or 17(R)-RvD1) has been shown to specifically block TRPV3. The *in vivo* administration of this compound has been shown to mitigate heat pain, a TRPV3-mediated sensory modality, but not mechanical pain under inflammatory conditions (Bang et al., 2012a). RvD1, which is relatively promiscuous in TRP interactions (as mentioned for TRPA1 above and TRPV4 below), has also been shown to inhibit TRPV3 channel opening and TRPV3-mediated pain (Bang et al., 2010). As shown in TRPA1 findings, AT-RvD1 and RvD1 unlikely utilize G-protein-coupled signaling for TRPV3 inhibition because these effects were resistant to treatment of the Gβγ inhibitor gallein and to treatment of the GPR agonists LL37 and WKYMVM (Bang et al., 2010, 2012a). Other indirect mechanisms such as participation of innate immune cells were also excluded by localizing RvDs to limited regions and times and showing that the effective doses were different than those for leukocytes (Krishnamoorthy et al., 2010).

TRPV4 Modulations by RvD1

Albeit to a lesser extent than TRPV1 and TRPA1, TRPV4 is also a polymodal sensor ion channel. Elevated temperatures (>27–34°C), mechanical stretching caused by pressure or swelling, and endogenous pro-inflammatory mediators such as epoxyeicosatrienoic acids (EETs) and dimethylallyl pyrophosphate are able to activate TRPV4 (Liedtke et al., 2000; Strotmann et al., 2000; Watanabe et al., 2003; Levine and Alessandri-Haber, 2007; Bang et al., 2012b). This activation is believed to lead to depolarization of the primary nociceptors, ultimately resulting in pain perception via the ascending transmission of this signal. While changes in thermonociceptive behaviors affected by TRPV4 knockout were significant but limited compared to phenotypes reported from genetic ablation of TRPV1 or TRPV3 (Todaka et al., 2004; Lee et al., 2005), the mechanosensory modalities that involve TRPV4 appear to be remarkable. Many types of inflammation caused somatosensory mechanical hyperalgesia or visceral mechanical hyperalgesia via TRPV4 activation (Alessandri-Haber et al., 2006; Chen et al., 2007; Grant et al., 2007; Brierley et al., 2008; Cenac et al., 2008). In the context of GPR-mediated inflammatory pain signaling, activation of protease-activated receptor 2 (PAR2, also known as GPR11) by trypsin or thrombin secreted from tissues is linked to TRPV4, which functions as its downstream effector for nociceptor neuronal firing (Grant et al., 2007). Histamine and serotonin also seem to contribute to GPR cascade, culminating in TRPV4 potentiation, thereby exacerbating visceral pain (Brierley et al., 2008; Cenac et al., 2008, 2010). In two different models of neuropathic pain, a chronic constriction injury model and a taxol-induced neuropathy model, TRPV4 contributed to increased mechanical pain (Alessandri-Haber et al., 2004; Zhang et al., 2008).

RvD1 is the only resolvin to date found to inhibit TRPV4. The Levine lab established an *in vivo* method for indexing TRPV4-specific somatosensory mechanical pain phenotypes. In this approach, hypotonic stimulation is applied locally to a rat paw when the paw is pre-primed with an inflammatory mediator prostaglandin E2 (Alessandri-Haber et al., 2003, 2006; Chen et al., 2007). The Hwang lab has reproduced this model with mice (Bang et al., 2010, 2012b). RvD1 was shown to be effective at alleviating TRPV4-specific inflammatory mechanical pain (Bang et al., 2010). Heat hyperalgesia in CFA-induced inflammation was also blunted by *in vivo* treatment of RvD1. Of the four heat sensor TRPs (TRPV1-4), TRPV3 and TRPV4 have been identified as molecular targets of RvD1. However, it remains unclear whether TRPV3 or TRPV4 predominates over the RvD1 effect on the thermal phenotype.

Using the same experimental approach described above for TRPA1 and TRPV3, an indirect ALX/FPR2-mediated GPR pathway has been excluded from the potential molecular interacting mechanism underlying these results. Although, it is unclear without binding assay, modulation via direct contact can be considered from what was recently hypothesized. It is suggested that chemical interactions between the lipid components of the bilayer membrane and the embedded regions of ion channel proteins are important for appropriate ion channel response and gating (Bavi et al., 2016). For ion channels with six transmembrane domains (which include TRP channel), one of the most critical regions in this regard is the intracellular linker between transmembrane domain (TM) 4 and TM5. For voltage-gated channels, this linker is crucial for allosteric conversion of movement of the TM4 voltage sensor upon depolarizing

stimulation into gating of the pore located between TM5 and TM6. According to recent structural approaches, the same linker of TRPV1 protein seems to bind to a lipidergic component and may also be responsible for transducing this binding energy to the pore region (Cao et al., 2013; Liao et al., 2013). The corresponding region of a bacterial mechanosensitive ion channel has been shown to be important since membrane stretching appears to alter binding between the linker and the bilayer lipids, resulting in pore opening (Bavi et al., 2016). Therefore, it is hypothesizable that the structure of this linker region is vulnerable (or sensitive) to external physical or chemical challenges (e.g., voltage increases that outwardly tilt the TM4 helix, membrane stretching that dysregulates bilayer contacts, lipidergic ligands that substitute a bilayer component, etc.), subsequently affecting the condition of the pore. In its closed states the linker region of TRPV1is pre-occupied by an endogenous lipid that has not yet been identified (Cao et al., 2013). In the structure of TRPV4, the closest homolog of TRPV1 (Teng et al., 2015), the lipid activators such as EETs may compete with the pre-occupying lipid, as predicted from capsaicin or HPETE interaction with TRPV1 (Hwang et al., 2000; Jordt and Julius, 2002). Conversely, RvD1 may more strongly stabilize the resting state than the pre-occupying endogenous one when located in the same place (**Figure 1**). When previous observations of lipidergic activators are considered together, it is possible that different double-bond locations of n−3 and n−6 derivatives may first direct stabilizing and destabilizing effects, and that the carbon lengths and locations of hydroxyl moieties that likely determine optimal occupation and hydrogen bond potential may define TRP specificity (Chyb et al., 1999; Hwang et al., 2000; Hu et al., 2006; Andersson et al., 2007; Komatsu et al., 2012; Motter and Ahern, 2012). Future experimental evidence is necessary to prove this hypothesis.

N-Methyl-D-Aspartic acid (NMDA) Receptor Modulation by Resolvins

Nociceptor populations among primary sensory neurons propagate peripheral pathologic signals, including those originating from inflammation, to the central nervous system. Moreover, these neurons actively contribute to pro-inflammatory processes via neurogenic inflammation mechanisms, by interactively exchanging various intercellular mediators with innervating tissues. These are important steps in the development of chronic inflammatory pain. In terms of pain relief, the TRPs mentioned above are essentially focused on when resolution mechanisms are examined, since the activation of these ion channels explains the outset of nociceptor excitation and pathologic hyperactivity, which often exacerbate neurogenic inflammation. The actions of pro-resolving lipids through these sensory TRP modulations all seem to converge on pain alleviation.

Postsynaptic dorsal horn neurons translate and relay signals received from the synaptic inputs of the central terminals of nociceptor neurons in the spinal cord (Choi et al., 2016). This synaptic transmission is the next gate to the development of persistent pain, which is determined by whether this spinal synapse is strengthened by a barrage of peripheral input. Postsynaptic NMDA receptor ion channels play a key role in this process (Lim et al., 2015). Pro-resolvents also down-regulate postsynaptic NMDA function in this central postsynapse, but partly in a different manner than for TRP channel modulations. The same GPR, chemR23, is involved in RvE1 action, but the dorsal horn-specific ERK phosphorylation cascade, which promotes NMDA potentiation, was mainly suppressed (Xu et al., 2010). A later RvD1 experiment has demonstrated another aspect of functional down-regulation of NMDA by showing that RvD1 treatment decreased phosphorylation of two NMDA subunits, NR1 and NR2B, in a model of pancreatitis-induced allodynia, but without confirming the knowledge of related GPRs (Quan-Xin et al., 2012).

Upon RvE1 exposure, presynaptic ERK phosphorylation has also been shown to be suppressed. In addition, the frequency of spontaneous excitatory postsynaptic potentials, which reflects not only postsynaptic function but also the extent of presynaptic glutamate release, was also decreased. This finding may also explain the RvE1-mediated suppression of TRPV1-independent presynaptic TNF-α signals (Xu et al., 2010). The mechanistic difference that occurs in the central synaptic region is conceivable while TRP channel modulation by pro-resolvents, which is probably more important in the periphery, is presumed to be mediated via direct binding or a rapid signal transduction through tightly coupled G-protein cycling.

PERSPECTIVES AND CONCLUSIONS

Here we reviewed the effects of various pro-resolving lipids on the functions of four sensory TRP channels and NMDA receptors that convey nociceptive signals. These pro-resolvents commonly showed strong inhibitory actions on these ion channels, leading to a final outcome of pain resolution. It is perhaps surprising that a group of endogenous lipid derivatives from common precursors can contribute to the resolution of two different pathologic processes, pain and inflammation, via differential cellular targets and biological mechanisms. Immunology studies continue to confirm that the absence of adverse effects from *in vivo* treatment of these substances. Based on current efficacy and safety information, translational applications of these substances to some specific diseases are eagerly anticipated. Before this promise can be realized, it would be advantageous to establish proof of concept to clarify the relevant molecular mechanisms. The most ambiguous ones to date are the binding mode by which these pro-resolving lipids interact with channel proteins and the mechanisms how they initiate GPR-mediated signal transduction. Although, one putative lipid-interacting site has been predicted, it has not yet been established whether resolvins modify channel activity through this site. When GPR coupled, it has not been completely answered which GPR predominantly drives the effect and which intracellular signal deactivates the channel. Most studies have focused on the sensory circuit. TRPV channels are involved in plastic changes in other brain synapses (Shibasaki et al., 2007; Gibson et al., 2008). NMDA receptors are essential ion channels for synaptic strengthening throughout

the whole brain. In addition to ion channel interaction in excitable neurons, changes in microglial function similar to those in monocyte/macrophage function may also be important checkpoints as once shown (Xu et al., 2010). Pro-resolvents are endogenously generated. Although, neuronal ion channels are strongly regulated by extraneous treatment, it remains to be elucidated which the major sources are among components in the nervous system and how similarly their transcellular biosyntheses occur as previously reported in the immune system.

Lessons from studies of the airway or other tissues may help to frame a different paradigm. In the airway research field, rather than channel activation or inhibition, changes in the expression of an ion channel are a more important axis to define the effect of a pro-resolvent, which have received little attention in the neuroscience field. The activity of Ca^{2+}-activated anion channels is increased upon pro-resolvent treatment in the airway epithelium (Verriere et al., 2012). These channels are considered to be an important temperature sensor and depolarizer in nociceptor neurons (Cho et al., 2012). However, this interaction has yet to be tested for neuronal outcomes. Future studies for all these questions will help determine whether these pro-resolvents (or future synthetic analogs thereof) will have clinical applications, how firmly the proof of concept needs to be established, and which pieces are missing in order to fully explain the molecular puzzle of ion channel interactions with lipidergic pro-resolvents.

AUTHOR CONTRIBUTIONS

GC analyzed documented results and prepared the preliminary draft. SH supervised the study and finalized the manuscript. All authors read and approved the final manuscript.

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Carbon Monoxide Modulates Connexin Function through a Lipid Peroxidation-Dependent Process: A Hypothesis

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Hemichannels are ion channels composed of six connexins (Cxs), and they have the peculiarity to be permeable not only to ions, but also to molecules such as ATP and glutamate. Under physiological conditions they present a low open probability, which is sufficient to enable them to participate in several physiological functions. However, massive and/or prolonged hemichannel opening induces or accelerates cell death. Therefore, the study of the molecular mechanisms that control hemichannel activity appears to be essential for understanding several physiological and pathological processes. Carbon monoxide (CO) is a gaseous transmitter that modulates many cellular processes, some of them through modulation of ion channel activity. CO exerts its biological actions through the activation of guanylate cyclase and/or inducing direct carbonylation of proline, threonine, lysine, and arginine. It is well accepted that guanylate cyclase dependent pathway and direct carbonylation, are not sensitive to reducing agents. However, it is important to point out that CO—through a lipid peroxide dependent process—can also induce a secondary carbonylation in cysteine groups, which is sensitive to reducing agents. Recently, in our laboratory we demonstrated that the application of CO donors to the bath solution inhibited Cx46 hemichannel currents in *Xenopus laevis* oocytes, a phenomenon that was fully reverted by reducing agents. Therefore, a plausible mechanism of CO-induced Cx46 hemichannel inhibition is through Cx46-lipid oxidation. In this work, I will present current evidence and some preliminary results that support the following hypothesis: Carbon monoxide inhibits Cx46 HCs through a lipid peroxidation-dependent process. The main goal of this paper is to broaden the scientific community interest in studying the relationship between CO-Fatty acids and hemichannels, which will pave the way to more research directed to the understanding of the molecular mechanism(s) that control the opening and closing of hemichannels in both physiological and pathological conditions.

Keywords: hemichannels, connexins, carbon monoxide, lipid peroxides, PUFAs

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INTRODUCTION

The hypothesis of the present work is: Carbon monoxide modulates connexin function through a lipid peroxidation-dependent process (**Figure 1**). This hypothesis is supported by the following knowledge and preliminary data.

GENERAL CHARACTERISTICS OF CONNEXINS

Connexins (Cxs) are transmembrane proteins that share a common topology: four transmembrane domains, two extracellular loops, one intracellular loop and the C, and N-termini located both on the cytoplasmic side. Twenty isoforms have been described in mammals (Reviewed in Eiberger et al., 2001), which are named following their expected molecular weight (e.g., Cx26 is expected to have an MW of ∼26 kDa). Even when Cx isoforms exhibit considerable homology, the C-terminus is the most variable region which differs in length and number of regulatory sites, which include consensus phosphorylation (Reviewed in Lampe and Lau, 2000), protein-protein interaction (Flores et al., 2008; Reviewed in Hervé et al., 2012) and cleavage sites (Lin et al., 1997). Almost all Cxs (except for Cx23; Lovine et al., 2008) have six conserved extracellular-loop cysteines (Cys), which have been proposed are essential for gap-junction channel (GJC) formation (Dahl et al., 1991). Finally, it is important to note that in mammals, virtually all cell types express at least one Cx type, but there are major differences in expression at the tissue level. Thus, for example, Cx43 is the most ubiquitously expressed (Beyer et al., 1987), whereas Cx46 has been described mostly in the lens (Paul et al., 1991). The widespread expression of Cxs suggests that they are essential for several physiological processes and that, due to their unique properties, they support cellular processes that cannot be replaced by any other Cx type (Wölfle et al., 2007).

CONNEXIN HEMICHANNELS

Hemichannels are composed of six Cxs monomers and are permeable to molecules up to ∼1.2 kDa. These ion channels participate in several physiological functions, such as spreading of calcium waves (Cotrina et al., 1998), Ca^{2+} permeation across plasma membrane (Sánchez et al., 2010; Schalper et al., 2010), cellular viability (Bellido and Plotkin, 2010), proliferation (Song et al., 2010), migration (Cotrina et al., 2008), light processing by the retina (Kamermans et al., 2001), mechanotransduction (Romanello et al., 2003), glucose uptake (Retamal et al., 2007), and synaptic plasticity (Stehberg et al., 2012), among others. Most of the hemichannel actions are exerted in part by the release of signaling molecules such as ATP (Stout et al., 2002), cyclic ADP-ribose (cADPR) (Bruzzone et al., 2001), prostaglandin E2 (PGE_2) (Cherian et al., 2005), glutamate, and aspartate (Ye et al., 2003) to the extracellular media, where they participate in paracrine/autocrine communication. On the other hand, under pathological conditions, massive and/or prolonged hemichannel opening induce and/or accelerate cell death. Nowadays, hemichannels with a gain of activity are known as "leaky hemichannels," and these have been observed in neurological disorders such as Charcot-Marie-Tooth disease, metabolic alterations such as ischemia, oculodentodigital dysplasia, skin diseases, inflammatory processes, and deafness (Reviewed in Retamal et al., 2015a). Although the mechanism by which hemichannels induce cell death is not well understood, it is highly probable that loss of metabolites (Stridh et al., 2008), ion gradients and membrane potential, as well as

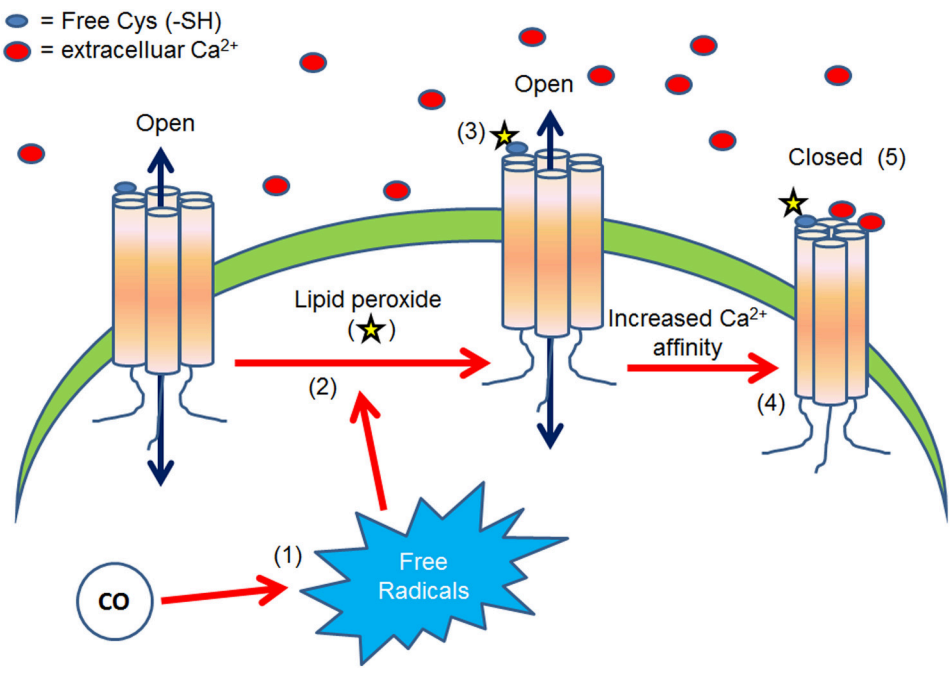

FIGURE 1 | The above diagram presents what is proposed as a possible molecular mechanism to explain the CO-induced Cx46 inhibition. CO increases free radicals concentration (1) which in turn induces lipid peroxide production (2). Then, lipid peroxides can induce the carbonylation of extracellular cysteine (-SH) (3), increasing Cx46-Ca^{2+} sensitivity (4) and thus stabilizing the closed state of the loop gating (5).

the massive entry of Ca^{2+} (Sánchez et al., 2010; Schalper et al., 2010) are some of the processes involved. Because hemichannels are important in cellular communication and cell survival, cells have several mechanisms to control hemichannel opening/closing, including phosphorylation (Bao et al., 2004), changes in membrane potential (Trexler et al., 1996; Retamal et al., 2010), alterations in extracellular Ca^{2+} concentration (Gómez-Hernández et al., 2003; Lopez et al., 2014), changes in redox potential (Retamal et al., 2006, 2009; Reviewed in Retamal, 2014) and presence of unsaturated fatty acids (Retamal et al., 2011). In summary, controlled hemichannel opening enables physiological autocrine/paracrine cell communication, but massive and/or uncontrolled hemichannel opening induces or accelerates cell death. Therefore, the study of the molecular mechanisms that control hemichannel activity is essential in order to understand several physiological and pathological processes.

HEMICHANNEL VOLTAGE GATING

Currently, it is well accepted that Cx channels are voltage dependent (Trexler et al., 1996; Retamal et al., 2010), although the molecular mechanisms that regulates the hemichannel voltage gating are not yet well understood. But, recently it was established by means of exchange different domains between Cx46 and Cx50 that the N-terminus contains the principal components of the hemichannel voltage sensor and unitary conductance (Kronengold et al., 2012). Additionally, there are two gating mechanisms that enable hemichannels to open and close, which are known as fast and slow/loop-gating (Reviewed in Oh and Bargiello, 2015). The first seems to depend on C-terminus docking to the intracellular loop and results in fast transitions from the open state to various substates (Reviewed in Oh and Bargiello, 2015). In contrast, slow or loop-gating is formed by the interface between the first transmembrane domain (TM1) and the first extracellular loop (EL1; Tang et al., 2009). Their distinctive feature is the slow time constant from fully open to fully closed states (tens to hundreds of milliseconds; Reviewed in Oh and Bargiello, 2015). Large conformational changes reduce the pore diameter from ∼20 Å to less than 4 Å (Verselis et al., 2009). It is widely accepted that extracellular divalent cations reduce the open probability of hemichannels in the plasma membrane (Gómez-Hernández et al., 2003; Lopez et al., 2014). Physiological concentrations of Ca^{2+} have been shown to stabilize the loop-gate closed state of Cx46 hemichannels (Verselis et al., 2009; Reviewed in Oh and Bargiello, 2015). Furthermore, atomic force microscopy studies of Cx26 hemichannels have indicated a narrowing of the extracellular channel entrance with 2 mM Ca^{2+} (Müller et al., 2002).

CARBON MONOXIDE

There are at least four gaseous transmitters, carbon monoxide (CO), nitric oxide (NO), hydrogen sulfide (H_2S; Reviewed in Farrugia and Szurszewski, 2014), and sulfur dioxide (SO_2; Chen et al., 2015), which are important modulators of the redox status and redox signaling. Under physiological conditions, CO is produced by two heme oxygenases (HO-1 and HO-2), which catalyze the catabolism of heme groups (Poss and Tonegawa, 1997). Both HO-1 and HO-2 are expressed in several cell types, and while HO-2 is constitutively expressed, HO-1 is inducible by several factors such as hypoxia and inflammation (Reviewed in Wu and Wang, 2005). Under physiological conditions, the human body produces 16.4 μmoles/h (Coburn, 1970), mainly by the action of HO-2 (Reviewed in Wu and Wang, 2005). Once CO is produced, it can be trapped by the hemoglobin, released by expiration (Reviewed in Wu and Wang, 2005) or act as a signaling molecule. In spite of the low concentration (nanomolar) of CO under physiological conditions, it has important roles in normal cardiac function, vascular contractility, platelet aggregation, monocyte activation, hypothalamic-pituitary-adrenal axis, odor response adaptation, nociception and chemoreception, among many other functions (for more details see, Wu and Wang, 2005). Additionally, CO production under physiological conditions can be increased by the induction of HO-1 controlled by physiological signaling molecules, such as transforming growth factor-β (Kutty et al., 1994), platelet-derived growth factor (Durante et al., 1999), and nitric oxide (Durante et al., 1997). Or, it can be decreased by angiotensin II (Ishizaka and Griendling, 1997). On the other hand, under pathological conditions HO-1 can be highly expressed and thus drastically increasing the CO levels (Reviewed in Wu and Wang, 2005). The expression of HO-1 has been implicated in diseases such as atherosclerosis, hypertension, transplant rejection, acute renal injury hyperoxia and hypoxia-induced lung injury, cancer, and neurodegeneration, among others diseases (Deshane et al., 2005; Reviewed in Wu and Wang, 2005). Although CO does not have free electrons as nitric oxide does, it can indirectly increase the oxidative stage of a cell. Thus, at high levels (>1000 ppm), CO increases protein and lipid oxidation (Reviewed in Wu and Wang, 2005), most likely as a result nitric oxide derived molecule production and dysregulation of GSH/GSSG relationship (Reviewed in Wu and Wang, 2005).

CO can act as a signaling molecule through two possible cellular pathways. First, the direct activation of guanylate cyclase, increases cGMP levels, which in turn activate PKG; and secondly, CO acts by direct carbonylation of amino acids, such as proline, threonine, lysine, and arginine (Reviewed in Cattaruzza and Hecker, 2008). However, CO can also induce an indirect carbonylation of cysteine residues through a lipid peroxidation dependent process (Reviewed in Wong et al., 2013). Thom (1990) showed that CO-dependent lipid peroxidation is reduced by the inhibition of xanthine oxidase or superoxide dismutase and iron chelators. Additionally, high concentration of CO was associated with increases in hydroxyl radical production and decreases in the reduced -oxidized glutathione ratio (GSH/GSSG; Lautier et al., 1992; Piantadosi et al., 1995; Reviewed in Wu and Wang, 2005). Therefore, CO can induce an oxidative intracellular environment, which in turn can favor the lipid peroxidation production rate. The process of lipid peroxidation is mediated through both enzymatic and non-enzymatic oxidation of poly

unsaturated fatty acids (PUFAs; Reviewed in Higdon et al., 2012). Enzymatic sources of lipid peroxides comprise both COXs (cyclo-oxygenases) that produce PG (prostaglandins) and LOXs (lipoxygenases) that produce leukotrienes (Reviewed in Higdon et al., 2012). On the other hand, non-enzymatic production is mediated by direct oxidation of PUFAs and comprises the production of 4-HNE (4-hydroxynonenal), malondialdehyde (MDA), and acrolein (Reviewed in Higdon et al., 2012). Interestingly, the secondary carbonylation of the Toll-like receptor by 4-HNE can be prevented by reducing agents (Kim et al., 2009), demonstrating that lipid peroxide-induced carbonylation is a dynamic process that can be modulated by the cellular redox status.

CARBON MONOXIDE MODULATES ION CHANNELS

In the early nineteens was described that mice exposure to high levels of CO-gas present degeneration of hippocampal CA1 pyramidal cells by a NMDA-dependent process, measured with hematoxylin-eosin staining (Ishimaru et al., 1992). This suggests that CO may induce neuronal cell death through changes of ion-channel activity. From this work, several reports strongly supported the notion that CO acts as an ion-channel modulator. Thus, it has been shown that CO increases the open probability of calcium-activated K^+ (KCa) channels in vascular smooth muscle cells (Wang et al., 1997) and human umbilical vein endothelial cells (Dong et al., 2007). The molecular mechanism of this phenomenon is not well understood, but it has been proposed that depends on the increase of the number of Ca^{2+} binding sites (Wang et al., 1997), expression of the alpha subunit (Wu et al., 2002), modulation by NO (Wang and Wu, 2003), and a metal-dependent like coordination of CO by Cys at position 911 (C911) (Williams et al., 2008; Telezhkin et al., 2011). Other ion channels are also affected by CO, such as a 70-pS K^+ channel in the thick ascending limb of Henle's loop (Liu et al., 1999), Kv2.1 (Dallas et al., 2011), hTREK-1 (Dallas et al., 2008), the amiloride-sensitive Na^+ channel (Althaus et al., 2009), Nav1.5 channels (Elies et al., 2014), Cav3.2 (Boycott et al., 2013), and P2X2 receptors (Wilkinson et al., 2009). In the case of Cav3.2 channels, CO induced-inhibition was dependent on the activation of an extracellular thioredoxin-dependent mechanism (Scragg et al., 2008). Together, these data suggest that CO exerts many of its effects through ion-channel modulation.

CARBON MONOXIDE MODULATES CX-HEMICHANNELS

Recently, was demonstrated that CO is a new hemichannel modulator (León-Paravic et al., 2014; Reviewed in Retamal et al., 2015b). The application of CO donors (CORM-A1, CORM-2, and CORM-3) to the bath solution inhibited the currents of Cx46 hemichannels expressed in *Xenopus laevis* oocytes (*X.* oocytes). The inhibition has an IC_{50} of approximated 3.4 μM, making Cx46 hemichannels an excellent CO sensor under pathological (>10 μM) condition (Kajimura et al., 2010). Moreover, CORM-2 effect was fully prevented by the addition of hemoglobin (a CO scavenger) to the bath solution and was correlated with Cx46 carbonylation, which in turn, produced important protein structural rearrangements *in vitro* (León-Paravic et al., 2014). Interestingly, the effect of CO did not involve changes in voltage dependency or modifications of the C-terminus. Additionally, hemichannels formed by Cx46 lacking extracellular-loop Cys were much less sensitive to CORM-2 compared to wild type Cx46 hemichannels. Moreover, hemichannel inhibition was fully recovered by addition of reducing agents to the bath solution (e.g., GSH and DTT; León-Paravic et al., 2014). The extracellular cysteine redox status potentially could affect the conformational disposition of the loop-gating, which in turn, is known to affect the loop- gating (Reviewed in Retamal et al., 2016). From these data it can be proposed that CO could inhibit Cx46 hemichannels through changes of the loop-gating properties, likely enhancing the effect of Ca^{2+} (**Figure 1**).

For many years, protein carbonylation was considered synonymous with protein degradation (Reviewed in Wong et al., 2013). However, recent evidence suggests that there is a naturally occurring process of protein decarbonylation (Wong et al., 2008; Reviewed in Wong et al., 2013). This mechanism involves an unknown thiol-dependent enzymatic process, in which the enzymes thioredoxin (Trx) and glutaredoxin (Grx1) seem to play important roles (Wong et al., 2008; Reviewed in Wong et al., 2013). Therefore, based on the current knowledge, the effect of CO (most likely secondary carbonylation) upon protein activity can be reversed and controlled by the redox status of a cell. Nevertheless, the exact molecular mechanism of decarbonylation is poorly understood. In our study (León-Paravic et al., 2014) we blocked TRx with aurarofin and a small recovery of hemichannel current was observed, which suggests that TRx does not play an important role in the recovery of hemichannel current induced by reducing agents. The question still remains as to whether GRx could participate. As indicated, CO can also act indirectly through lipid peroxidation of Cys groups (Reviewed in Wong et al., 2013; Milic et al., 2015). Lipid-induced protein oxidation can be reverted by glutathione peroxidase (GPx4) and glutathione S-transferase (GST; Reviewed in Ribas et al., 2014), which are activated by reducing agents (Reviewed in Ribas et al., 2014). Therefore, a plausible mechanism of CO-induced Cx46 hemichannel inhibition is through Cx46-lipid oxidation.

In support of the role of oxidized lipids on Cx46 hemichannel inhibition, polyunsaturated fatty acids (PUFAs), such as linoleic acid and arachidonic acids, which can be easily oxidized (Reviewed in Ribas et al., 2014), inhibits Cx46 hemichannels *in vitro* (Retamal et al., 2011). Therefore, it cannot be ruled out that PUFAs may exert their inhibitory effects upon hemichannels through their oxidized-derived molecules. Moreover, preliminary results have shown that 4-Hydroxy-2-nonenal (4-HNE), a reactive aldehyde derived from oxidized lipids (100 μM), inhibits by 60 ± 12% Cx46 hemichannels in *X.* oocytes (**Figure 2**) and Vitamin C—a lipid peroxide inhibitor—reduced by ∼50% the effect of CO upon Cx46 hemichannels (**Figure 2**). Moreover, the presence of Ca^{2+} in the

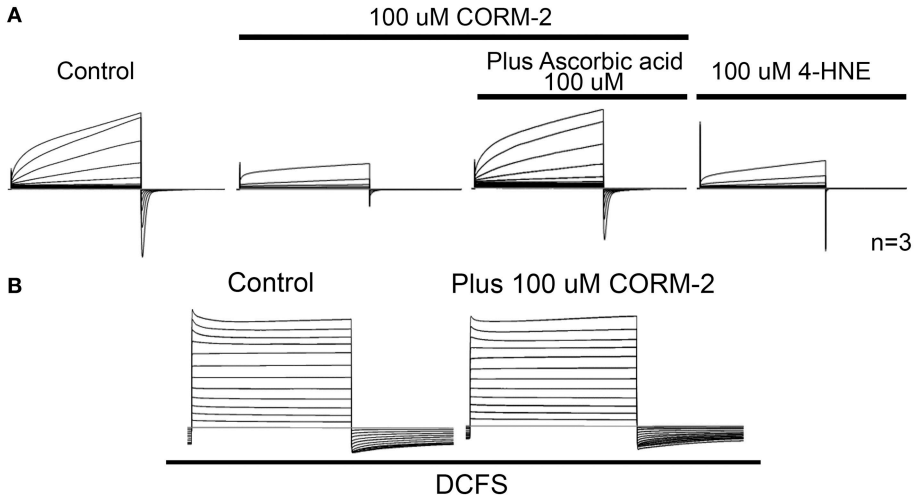

FIGURE 2 | CO effect appears to be mediated by lipid peroxides. (A) Representative control of Cx46 hemichannel currents in *Xenopus laevis* oocytes recorded in ND96 solution (containing 1.8 mM Ca^{2+} and 1.0 mM Mg^{2+}) by means of dual whole cell voltage clamp technique. The presence of 100 μM CORM-2 induces a dramatic drop in the current amplitude. Most of the inhibition induced by CORM-2 was prevented by the co-addition of 100 μM ascorbic acid to the bath solution. This suggests that the effect of CO needs free radical production into the *Xenopus* oocytes. In parallel experiments, oocytes expressing Cx46 were exposed to the lipid peroxide 4-HNE (100 μM), and an evident hemichannel current inhibition was observed. $n = 3$ for each condition. **(B)** Representative recordings of oocytes expressing Cx46 placed in a DCFS, without (control) or with 100 μM CORM-2 ($n = 3$).

extracellular media is fundamental for observing CO-induced inhibition of Cx46 hemichannels, suggesting that the CO-induced inhibition/extracellular Cys-lipid peroxidation involve certain conformational changes that alter loop gating properties (**Figure 2**).

FUTURE DIRECTIONS

Hemichannels are relevant players in the development and progression of several diseases, and they are now used as targets for developing new molecules for disease treatments (Reviewed in Retamal et al., 2015a). However, in spite of years of research, the molecular mechanisms that control the opening and closing of these channels are still not well understood. Thus, it is highly relevant to understand these mechanisms and project this knowledge to produce new agonist(s)/antagonist(s) against Cx- hemichannels, as well as to understand why hemichannels become lethal under certain pathological conditions. Although the effect of CO upon GJC has not been studied, it is possible to propose that CO may not have a relevant impact in GJC properties. It because, CO/lipid peroxides seem to act through modifications of extracellular Cys, which in GJC are not accessible for modifications by reducing nor oxidant molecules.

CO has been used for the treatment of several diseases, but many of its effects are far from being well understood. Therefore, the current knowledge is insufficient for understanding how CO exerts its action at the cellular level and, thus, to find possible side effects of this treatment. Also, this knowledge may help to develop new strategies in the therapeutic use of CO, e.g., under metabolic stress, where hemichannels become massively open, which accelerates cell death. Therefore, a possible application of this research would be to pursue the use of CO as a hemichannel inhibitor in preventing or limiting stroke-induced cell death.

AUTHOR CONTRIBUTIONS

MR wrote this paper and did all the figures.

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