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Antithetical modes of and the Ca^{2+} sensors targeting in ANF-RGC and ROS-GC1 membrane guanylate cyclases

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The membrane guanylate cyclase family has been branched into three subfamilies: natriuretic peptide hormone surface receptors, Ca^{2+}-modulated neuronal ROS-GC, and Ca^{2+}-modulated odorant surface receptor ONE-GC. The first subfamily is solely modulated by the extracellularly generated hormonal signals; the second, by the intracellularly generated sensory and sensory-linked signals; and the third, by combination of these two. The present study defines a new paradigm and a new mechanism of Ca^{2+} signaling. (1) It demonstrates for the first time that ANF-RGC, the prototype member of the surface receptor subfamily, is stimulated by free $[Ca^{2+}]_i$. The stimulation occurs *via* myristoylated form of neurocalcin *δ*, and both the guanylate cyclase and the calcium sensor neurocalcin δ are present in the glomerulosa region of the adrenal gland. (2) The EF-2, EF-3 and EF-4 hands of GCAP1 sense the progressive increment of $[Ca^{2+}]_i$ and with a $K_{1/2}$ of 100 nM turn ROS-GC1 "OFF." In total reversal, the same EF hands upon sensing the progressive increment of $[Ca^{2+}]_i$ with $K_{1/2}$ turn ONE-GC "ON." The findings suggest a universal Ca^{2+}-modulated signal transduction theme of the membrane guanylate cyclase family; demonstrate that signaling of ANF-RGC occurs by the peptide hormones and also by $[Ca^{2+}]_i$ signals; that for the Ca^{2+} signal transduction, ANF-RGC functions as a two-component transduction system consisting of the Ca^{2+} sensor neurocalcin δ and the transducer ANF-RGC; and that the neurocalcin δ in this case expands beyond its NCS family. Furthermore, the study shows a novel mechanism of the $[Ca^{2+}]_i$ sensor GCAP1 where it acts as an antithetical NCS for the signaling mechanisms of ROS-GC1 and ONE-GC.

Keywords: calcium, GCAP1, neurocalcin δ, neuronal calcium sensors, membrane guanylate cyclase, cyclic GMP, atrial natriuretic factor receptor membrane guanylate cyclase, olfactory neuroepithelium membrane guanylate cyclase

INTRODUCTION

Ca^{2+} sensor proteins form a group of Ca^{2+} binding proteins that, in defined concentrations of free intracellular Ca^{2+}, function as modulators of the activities of specific target proteins. They acquire these modulatory abilities by binding Ca^{2+} through specific helix-loop-helix structural motifs called EF hands. Binding of Ca^{2+} to the EF hand motif triggers conformational changes in the respective Ca^{2+} sensor protein that enable it to perform Ca^{2+}-dependent functions ranging from regulation of ion channels permeability to gene expression, cellular survival and apoptosis (reviewed in Bhattacharya et al., 2004; Braunewell, 2005). Neuronal Ca^{2+} sensor (NCS) proteins constitute a subfamily of Ca^{2+} sensor proteins and were initially considered to be expressed exclusively in neurons, but now are found in other tissues as well. NCS proteins are subclassified into five groups based on their sequence similarities. In mammals, 14 conserved NCS proteins exist (reviewed in Burgoyne, 2007) and all of them have 4 EF hand Ca^{2+} binding motifs but inactivating amino acid substitutions make the first EF hand non-functional for Ca^{2+} binding. In addition to the first inactive EF hand, recoverin and K^+-channel interacting protein type1 (KChIP1) harbor another non-functional EF hand. Except for KChIP2, 3 and 4 all mammalian NCS proteins contain a consensus sequence for N-terminal myristoylation (reviewed in Burgoyne and Weiss, 2001; Burgoyne et al., 2004). Some isoforms of KChIP2 and 3 are possibly palmitoylated (Takimoto et al., 2002). Both myristoylation and palmitoylation of NCS proteins allows for some of them membrane association, either permanently or transiently in response to changes in the intracellular Ca^{2+} concentration. For GCAP1 and NCS-1, however, Ca^{2+} plays no role in membrane attachment of the protein (Hwang and Koch, 2002; O'Callaghan et al., 2002; Orban et al., 2010).

Neurocalcin δ together with visinin-like proteins (VILIPs) and hippocalcin form a distinct subfamily of NCS proteins. It is acylated at the N-terminus by myristic acid and undergoes a classical calcium-myristoyl switch (Ladant, 1995) e.g. it buries the myristoyl group in a hydrophobic pocket in a Ca^{2+}-free form and expose it in Ca^{2+}-bound form as it is observed for recoverin (Zozulya and Stryer, 1992). However, once it binds in a Ca^{2+}-dependent fashion to the membrane phospholipids part of it remains membrane bound even after removing Ca^{2+} by the addition of EGTA (Krishnan et al., 2004). Although the highest level of neurocalcin δ has been detected in neuronal tissues, its expression level in peripheral tissues is also significant. Functionally,

neurocalcin δ has been linked to receptor endocytosis through interaction with α- and β-clathrin and β-adaptin (Ivings et al., 2002), trafficking and membrane delivery of glutamate receptors of the kainate type (Coussen and Mulle, 2006), and due to its Ca^{2+}-dependent affinity for S100B protein and tubulin β-chain (Okazaki et al., 1995), with microtubule assembly (Iino et al., 1995). In the sensory and sensory-linked neurons, the presence of neurocalcin δ has been found in the inner plexiform layer of the retina, *e.g.* in the amacrine and ganglion cells (Krishnan et al., 2004), olfactory sensory neurons (Duda et al., 2001, 2004) and very recently, it has been identified in type II cells of mouse circumvallate taste papillae, indicating its possible role in the gustatory transduction (Rebello et al., 2011).

Further, neurocalcin δ can act as Ca^{2+}-dependent modulator of membrane guanylate cyclase ROS-GC1 in the retina and ONE-GC, in the olfactory neuroepithelium. There, it co-localizes with its respective target guanylate cyclases (Duda et al., 2001, 2004; Krishnan et al., 2004). The exact physiological significance of the ROS-GC1-neurocalcin δ signaling system in the retinal neurons is not known yet, it has, however, been proposed that the system may be involved in synaptic processes (Krishnan et al., 2004). In the olfactory neuroepithelium neurocalcin δ serves as a Ca^{2+} sensor component of the two-step odorant uroguanylin signaling machinery (Duda and Sharma, 2009).

Guanylate cyclase activating protein type 1 (GCAP1) is a well characterized member of the NCS proteins subfamily (reviewed in Palczewski et al., 2004; Sharma et al., 2004; Behnen et al., 2010; Koch et al., 2010). Like other homologs and orthologs of the subfamily, it harbors four EF hand Ca^{2+} binding motifs, of which the first one is inactive. GCAP1 is acylated at the N-terminus by myristic acid that is buried in a hydrophobic pocket (Stephen et al., 2007) and changing Ca^{2+} concentrations do not trigger exposure of the myristoyl group (Orban et al., 2010). Instead the myristoyl group remains buried in a hydrophobic cavity. Thus, GCAP1 does not interact with the membranes in a Ca^{2+}-dependent fashion (Hwang and Koch, 2002; Haynes and Burgoyne, 2008) and does not undergo a classical calcium myristoyl switch. Identified first in the retinal photoreceptors (Gorczyca et al., 1994; Frins et al., 1996), GCAP1 transmits Ca^{2+} signals to and controls the activity of rod outer segment guanylate cyclase, ROS-GC. It activates ROS-GC in the absence of Ca^{2+}, when immediately after illumination the cytoplasmic Ca^{2+} drops in a photoreceptor cell. GCAPs are thought to switch to a Ca^{2+}-free but Mg^{2+}-bound state, which represents the activating form (Peshenko and Dizhoor, 2006). Increasing concentrations of free Ca^{2+} diminish the activation process leading even to an inhibition below the basal cyclase activity level (Duda et al., 1996). This regulatory process of ROS-GC is essential for the photoresponse recovery of visual cells (Mendez et al., 2001; Howes et al., 2002).

Here, new observations on Ca^{2+}-dependent modes of neurocalcin δ and GCAP1 in modulating membrane guanylate cyclase signaling are presented. The results disclose a new model of Ca^{2+}-neurocalcin δ signaling of ANF-RGC and a new signaling mechanism of GCAP1 in which it serves as an antithetical Ca^{2+} sensor in the phototransduction and the olfactory sensory neurons. Thus, they indicate an increasing complexity in the regulatory modes of membrane guanylate cyclases and this enables them to perform multiple cellular functions.

MATERIALS AND METHODS

Mutagenesis. Point mutations in ONE-GC and GCAP1 cDNA were introduced using Quick Change mutagenesis kit (Stratagene) and appropriate mutagenic primers. ONE-GC $F^{585}S$ mutation-Forward primer 5′-TGGCTGAAGAAGTCTGAGGCAGGC ACG-3′; Reverse primer 5′-CGTGCCTGCCTCAGACTTCTTCAG CCA-3′ (the mutated sequence is underlined). Construction of the GCAP1($D^{100}E$) mutant is described in detail in (Kitiratschky et al., 2009). The mutants were verified by sequencing. To construct the ANF-RGC Ext^{-} mutant two Hpa1 restriction sites were introduced at nucleotide positions 437–442 (Forward primer 5′-GTGGTGCTGCCGCTG GTAAACAACACCTCGTACCCG-3′, Reverse primer 5′-CGG GTACGAGGTGTTTACCAGCGGCAGCACCAC-3′; Hpa1 recognition sequence is underlined) and at nucleotide positions 1697–1702 (Forward primer 5′-AATGAGGACCCAG CCGTAAACCAAGACCACTTT-3′; Reverse primer 5′-AAAGTG GTCTTGGTTTACGGCTGGGTCCTCATT-3′; Hpa1 recognition sequence is underlined). The 1242 bp fragment was excised and the remaining part was religated.

Expression in COS cells. COS7 cells (simian virus 40-transformed African green monkey kidney cells) were maintained in DMEM medium supplemented with 10% fetal bovine serum and penicillin, streptomycin antibiotics on 10 cm diameter cell culture dishes in humidified atmosphere of 95% O_2/5% CO_2. At approximately 65% confluency the cells were transfected with 25 μg of appropriate plasmid cDNA using calcium phosphate co-precipitation technique (Sambrook et al., 1989). In control experiments the cells were transfected with 25 μg of empty expression vector. 64 h after transfection cells were washed with 50 mM Tris-HCl pH 7.4/10 mM Mg^{2+} buffer, homogenized and the particulate fraction pelleted by centrifugation.

Guanylate cyclase activity assay. The membranes were incubated on ice-bath with or without GCAP1 or neurocalcin δ in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg creatine kinase and 50 mM Tris-HCl, pH 7.5. Appropriate Ca^{2+} concentrations were adjusted with pre-calibrated Ca^{2+}/EGTA solutions (Molecular Probes). The total assay volume was 25 μl. The reaction was initiated by addition of the substrate solution (4 mM $MgCl_2$ and 1 mM GTP, final concentration) and maintained by incubation at 37°C for 10 min. The reaction was terminated by the addition of 225 μl of 50 mM sodium acetate buffer, pH 6.2 followed by heating on a boiling water bath for 3 min. The amount of cyclic GMP formed was determined by radioimmunoassay (Nambi et al., 1982).

Expression and purification of GCAP1, GCAP1($D^{100}E$) and neurocalcin δ. GCAP1 was expressed and purified as in (Duda et al., 1999), GCAP1($D^{100}E$) as in (Kitiratschky et al., 2009), and neurocalcin δ as in (Duda et al., 2004).

Antibodies. The specificity of antibody against neurocalcin δ has been described previously (Duda et al., 2004). Antibody against ANF-RGC was raised against the kinase homology domain in rabbits. Specificity of the antibody was tested through Western blot using membranes of COS cells expressing all three

receptor guanylate cyclases, ANF-RGC, CNP-receptor guanylate cyclase (CNP-RGC) and enterotoxin receptor guanylate cyclase (STa-RGC). The antibody recognized only ANF-RGC (data not shown). The antibodies were affinity purified. Secondary antibodies conjugated to a fluorescent dye (DyLight 488 and DyLight 549) were purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.

Immunohistochemistry. Mice were sacrificed by lethal injection of ketamine/xylazine (the protocol approved by the Salus University IUCAC) and perfused through the heart, first with a standard Tris-buffered saline (TBS) and then with freshly prepared 4% paraformaldehyde in TBS. The adrenal glands were removed and fixed for 1–4 h in 4% paraformaldehyde with TBS at 4°C, cryoprotected in 30% sucrose overnight at 4°C and cut into 20 μm sections using Hacker-Bright OTF5000 microtome cryostat (HACKER Instruments and Industries Inc., Winnsboro, SC). The sections were washed with TBS, blocked in 10% normal serum in TBS/0.5% Triton X-100 (TTBS) for 1 h at room temperature, washed with TTBS, incubated with respective antibody in blocking solution overnight at 4°C, washed with TTBS for and then incubated with DyLight conjugated donkey anti-rabbit antibody (200:1) for 1 h, washed with TTBS. Images were acquired using an inverted Olympus IX81 microscope/FV1000 Spectral laser confocal system, and analyzed using Olympus FluoView FV10-ASW software. Digital images were processed using Adobe Photoshop software.

Western blot. After boiling in a gel-loading buffer [62.5 mM Tris-HCl, (pH 7.5), 2% SDS, 5% glycerol, 1 mM β-mercaptoethanol (βME), and 0.005% bromophenol blue] the proteins (membranes of transfected COS cells or mouse adrenal gland homogenate) were subjected to SDS-polyacrylamide gel electrophoresis in a buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. The proteins were transferred to immobilon membranes (Millipore) in the same buffer but containing 5% methanol. The blot was incubated in (TBS pH 7.5) containing 100 mM Tris-HCl, 0.9% NaCl, and 0.05% Tween-20 (TBS-T) with 5% powdered non-fat Carnation milk (blocking buffer) overnight at 4°C and rinsed with TBS-T. The antibodies were added to the solution and the incubation continued for 1 h at room temperature. After the blot was rinsed with with TBS-T, the incubation was continued with the secondary antibody conjugated to horseradish peroxidase in blocking buffer for another hour. Finally, the blot was treated with SuperSignal^{R} West Pico chemiluminescent substrate (Thermo Scienitifc; according to the manufacturer's protocol). The immunoreactive band was visualized by exposing the blot to Kodak X-ray film.

RESULTS AND DISCUSSION

ANF RECEPTOR GUANYLATE CYCLASE, ANF-RGC, IS MODULATED BY Ca^{2+} SIGNALS

Neurocalcin δ transmits Ca^{2+} signal to ANF-RGC

Based on almost three decades of research the family of mammalian membrane guanylate cyclases has been firmly divided into two subfamilies, receptor guanylate cyclases and intracellular Ca^{2+} regulated guanylate cyclases. The first group included the receptor for natriuretic factor type A (ANF) and type B (BNP) guanylate cyclase ANF-RGC, the receptor for type C natriuretic peptide guanylate cyclase CNP-RGC, and heat-stable enterotoxin (and also guanylin and uroguanylin) receptor guanylate cyclase STa-RGC; the second group is comprised of the photoreceptor guanylate cyclases ROS-GC1 and ROS-GC2 and the olfactory neuroepithelium guanylate cyclase ONE-GC.

To determine whether a receptor guanylate cyclase could also respond to Ca^{2+} signals transmitted to it through a calcium sensor protein, the prototype receptor cyclase ANF-RGC and NCS protein neurocalcin δ were chosen. A clue for selecting neurocalcin δ was the observation that it targets the conserved membrane guanylate cyclase catalytic domain of ROS-GC1.

Membranes of COS cells expressing recombinant ANF-RGC were incubated with series of increasing concentrations of purified myristoylated neurocalcin δ at a fixed 10 μM Ca^{2+}. No extracellular ligand of ANF-RGC, ANF or BNP, was added to the reaction mixture. ANF-RGC activity was stimulated in the neurocalcin δ concentration-dependent manner; half-maximal activation of the cyclase occurred at ∼0.5 μM and the maximal activation of 4.8-fold above the basal value was observed at 2 μM myristoylated neurocalcin δ (**Figure 1**: closed circles). The calculated Hill's coefficient for the stimulatory effect was 2.1 ± 0.5. In the membranes of cells transfected with the vector alone the cyclase activity was negligible, 0.2 pmol cyclic GMP $min^{-1}(mg\ protein)^{-1}$ (**Figure 1**: closed diamonds) and was unaffected by neurocalcin δ in the presence of Ca^{2+}. To verify that the observed effect of Ca^{2+}-neurocalcin δ is specific the cyclase activity was measured in the presence of neurocalcin δ but in the absence of Ca^{2+} (1 mM EGTA was added to the reaction mixture). The absence of Ca^{2+} did not affect the basal ANF-RGC activity; it was 12 ± 2 pmol cyclic GMP $min^{-1}(mg\ protein)^{-1}$

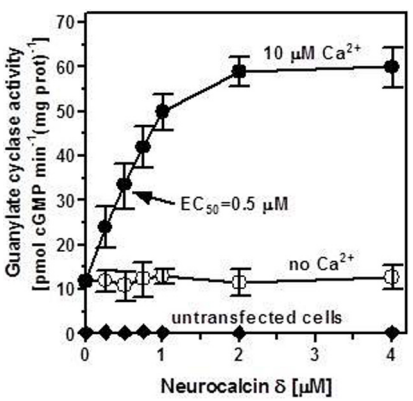

FIGURE 1 | Ca^{2+}-bound neurocalcin δ stimulates ANF-RGC activity. COS cells were transfected with ANF-RGC cDNA and their membrane fraction was analyzed for neurocalcin δ-dependent cyclase activity in the absence (open circles) and presence of 10 μM Ca^{2+} (closed circles). COS cells transfected with an empty vector were analyzed identically (closed diamonds). The extracellular hormone ligand of ANF-RGC, the atrial natriuretic factor (ANF) was absent from the reaction mixture. The experiment was done in triplicate and repeated four times. The results shown are average ± SD from these experiments. The EC_{50} value was determined graphically. Neurocalcin δ used was myristoylated. The myristoylated form of neurocalcin δ was expressed and purified as described in Krishnan et al. (2004).

in the presence of 10 μM Ca^{2+} and 11.9 ± 1.8 pmol cyclic GMP min^{-1}(mg protein)$^{-1}$ in the presence of 1 mM EGTA. In the absence of Ca^{2+} neurocalcin δ, however, did not stimulate ANF-RGC activity (**Figure 1**: open circles). Thus, ANF-RGC activity is not only regulated by ANF or BNP; *in vitro* it is also regulated by myristoylated neurocalcin δ in the presence of Ca^{2+}.

Neurocalcin δ targets the intracellular domain of ANF-RGC

It is well established that ANF and BNP, the hormone-ligands of ANF-RGC, signal through the cyclase's extracellular domain (Duda et al., 1991; Ogawa et al., 2004; reviewed in Sharma, 2002, 2010). Neurocalcin δ, on the other hand, is an intracellular protein, therefore the respective target sites of these two types of ligand, ANF/BNP and neurocalcin δ, reside on the opposite sites of the transmembrane domain of ANF-RGC. To determine the biochemical requirements for neurocalcin δ effect on ANF-RGC activity namely, whether the isolated intracellular portion of ANF-RGC is sufficient for neurocalcin δ to exhibit its stimulatory effect or whether the intact ANF-RGC protein is necessary, an ANF-RGC deletion mutant was prepared in which the extracellular receptor domain (aa 12–433) was deleted. The mutant, however, had retained the leader sequence to ensure its proper membrane targeting. This mutant was transiently expressed in COS cells and their membranes were appropriately treated with ANF or myristoylated neurocalcin δ and 10 μM Ca^{2+}. Both proteins had comparable basal guanylate cyclase activities, 13 and 12.2 pmol cyclic GMP min^{-1}(mg protein)$^{-1}$ for the full-length ANF-RGC and the deletion mutant, respectively. As expected, the mutant was unresponsive to ANF (**Figure 2**: open circles), however, neurocalcin δ stimulated its activity in a dose-dependent fashion (**Figure 2**: closed diamonds). The stimulatory profile was indistinguishable from that of the full-length ANF-RGC (**Figure 2**: closed circles). Also the Hill's coefficients for the neurocalcin δ effect on both cyclases were identical 2.1 ± 0.5 and 2.05 ± 0.4 for the full-length ANF-RGC and for the deletion mutant, respectively. It is, therefore, concluded that the extracellular domain has no structural role in ANF-RGC ability to respond to and be stimulated by myristoylated neurocalcin δ.

ANF-RGC and neurocalcin δ co-exist in the glomerulosa cells of the adrenal gland

Are these *in vitro* biochemical findings on ANF-RGC activity modulation by Ca^{2+} *via* neurocalcin δ of physiological relevance? A first hint could be the co-expression of both proteins in the same tissue, organ or cell type. Guided by intuition the mouse adrenal gland was analyzed for the presence of ANF-RGC and neurocalcin δ, first by Western blot and then through immunocytochemistry.

The gland was homogenized and the homogenate after SDS-polyacrylamide gel electrophoresis was analyzed for the presence of ANF-RGC or neurocalcin δ by Western blot. As shown in **Figure 3**, with antibody against ANF-RGC as a probe (panel "ANF-RGC") an intense immunoreactive band was observed at expected molecular weight of ∼130 kDa. With neurocalcin δ antibody as a probe (**Figure 3**: panel "NCδ") the presence of an immunoreactive protein with mobility of ∼23 kDa corresponding to the molecular weight of neurocalcin δ was observed. These results show that both ANF-RGC and neurocalcin δ are expressed in the mouse adrenal, they, however, do not provide information whether these proteins are expressed in the same type of adrenal gland cells. To determine whether these proteins co-localize in the

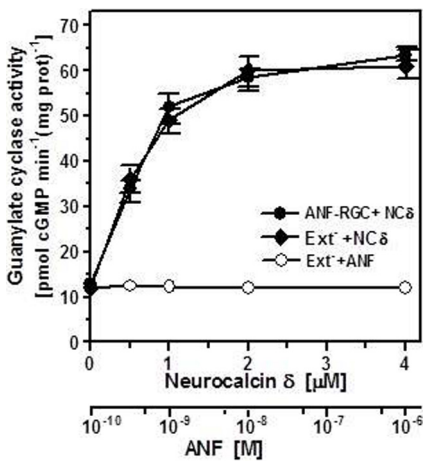

FIGURE 2 | The intracellular portion of ANF-RGC is sufficient for neurocalcin δ and Ca^{2+} to stimulate ANF-RGC activity. The ANF-RGC deletion mutant lacking the extracellular receptor domain aa12–433, was constructed and expressed in COS cells. The particulate fraction of these cells was assayed for guanylate cyclase activity in the presence of increasing concentrations of myristoylated neurocalcin δ and 10 μM Ca^{2+} or 0.5 mM ATP and increasing concentrations of ANF. Membranes of COS cells expressing full-length ANF-RGC were processed in parallel as positive control. The experiment was performed in triplicate and repeated two times. The results shown are mean ± SD from these experiments.

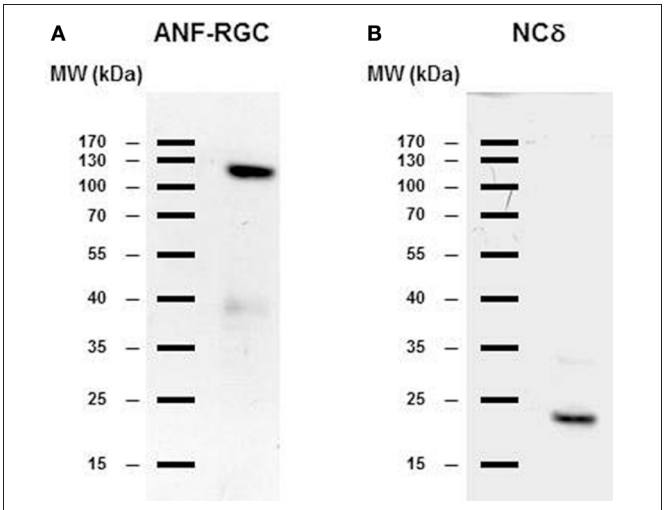

FIGURE 3 | ANF-RGC and neurocalcin δ are expressed in the mouse adrenal gland. Mouse adrenal gland was homogenized in 50 mM Tri-HCl/10 mM $MgCl_2$ buffer (pH 7.5) containing protease inhibitor cocktail (Sigma). The proteins (∼40 μg/lane) were subject to SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using antibody against ANF-RGC or neurocalcin δ as described in the "Materials and Methods" section. **(A)** immunoreactivity with ANF-RGC antibody. **(B)** immunoreactivity with neurocalcin δ antibody.

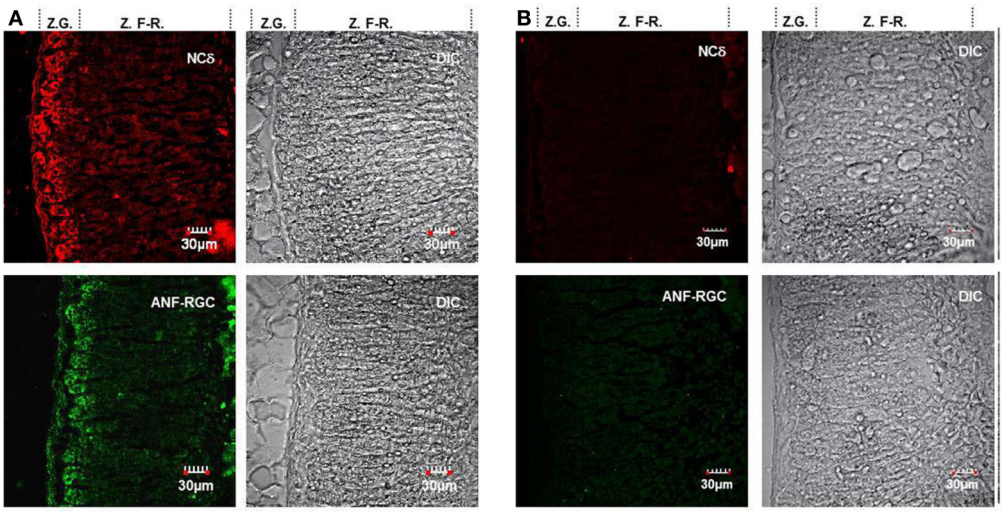

FIGURE 4 | ANF-RGC and neurocalcin δ are co-expressed in the glomerulosa cells of mouse adrenal gland. (A) Serial cryosections of the mouse adrenal gland were immunostained with neurocalcin δ (panel "NCδ") or ANF-RGC (panel "ANF-RGC") antibodies. The DIC image showing the integrity of the adrenal gland sections are presented at the left ("DIC"). "Z.G." and "Z.F-R." denote zona glomerulosa and zona fasciculata-reticularis, respectively. Intense staining with either antibody was observed in the zona glomerulosa. **(B)** The immunostaining with the primary antibodies is specific. The mouse adrenal gland cryosections were processed identically as in **(A)** except that the primary antibody for ANF-RGC or neurocalcin δ was omitted in the respective incubation mixture but incubation with fluorescently labeled secondary antibody was carried out as in **(A)**. The DIC image showing the integrity of the sections are presented ("DIC").

adrenal gland immunocytochemical analyzes were carried out. Sections of the adrenal gland were immunostained with specific antibodies against ANF-RGC and neurocalcin δ. Because both antibodies used were raised in rabbits, co-immunostaining was not feasible, therefore, staining of consecutive sections was performed. The results are shown in **Figure 4A**. Intense staining with anti neurocalcin δ antibody was observed in the adrenal zona glomerulosa (**Figure 4A**: panel "NCδ" Z.G.). Also, strong immunoreactivity with ANF-RGC antibodies was observed in zona glomerulosa (**Figure 4A**: panel "ANF-RGC" Z.G.). This localization of ANF-RGC within the mouse adrenal gland is consistent with the protein's localization in the bovine adrenal gland (Meloche et al., 1988). To verify the specificity of the staining, in control reactions the primary antibodies were omitted but secondary antibody was added. Without the primary antibodies there was no specific staining in the sections analyzed (**Figure 4B**: control). It was therefore concluded that both ANF-RGC and neurocalcin δ co-exist in the adrenal glomerulosa cells. Although some faint staining in both sections was observed for the fasciculate-reticularis cells (Z.F-R. region in both "NCδ" and "ANF-RGC" panels), without detailed co-localization experiments it is not possible to conclude on ANF-RGC and neurocalcin δ co-existence there.

The second question asked was: are ANF-RGC and neurocalcin δ co-existing in the adrenal gland functionally linked? Mouse adrenal gland was homogenized in the presence of 1 mM EGTA or 10 μM Ca^{2+}, the particulate fraction was prepared and analyzed for guanylate cyclase activity. In the absence of Ca^{2+} the activity was 70 ± 9 pmol cyclic GMP min^{-1} (mg protein)$^{-1}$ and in the presence of Ca^{2+}, 245 ± 28 pmol cyclic GMP min^{-1}(mg protein)$^{-1}$ (**Figure 5**). At this stage it is not possible to conclude with certainty, however, if native neurocalcin δ in the adrenal gland is pre-bound to ANF-RGC or if it is undergoing a Ca^{2+} mirystoyl switch and interacts with ANF-RGC in a reversible manner, the guanylate cyclase activity measured at 10 μM Ca^{2+} reflects the neurocalcin δ-stimulated ANF-RGC activity.

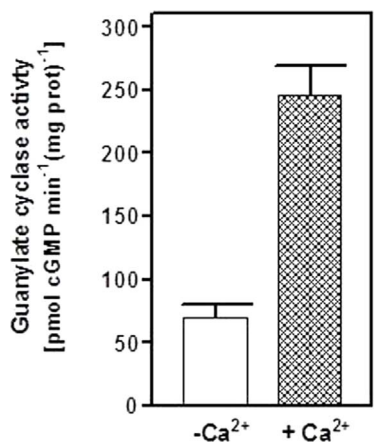

FIGURE 5 | Neurocalcin δ stimulates ANF-RGC in membranes of mouse adrenal gland. Mouse adrenal gland was homogenized in Tris-Mg^{2+} buffer pH 7.4 with or without 10 μM Ca^{2+}. The particulate fractions were prepared from each homogenate and assayed for guanylate cyclase activity. The experiment was done in triplicate and repeated two times. The results shown are average ± SD from these experiments.

What is the physiological significance of neurocalcin δ modulation of ANF-RGC activity in the adrenal zona glomerulosa? At this moment there is no definite answer to this question. A clue, however, may be provided by the facts that the adrenal glomerulosa cells are the site of aldosterone synthesis, that aldosterone synthesis is triggered by the increase in cytosolic Ca^{2+} concentration, and that the ANF-RGC activity offsets the renin-angiotensin-aldosterone system and inhibits aldosterone synthesis (Burnett et al., 1984; Brenner et al., 1990; Aoki et al., 2000; Shi et al., 2001). Because a measurable time is necessary for hormonal (ANF) turning "ON" the ANF-RGC signal transduction system resulting in the inhibition of aldosterone synthesis, it is tempting to hypothesize that before the hormonal ANF signal is activated, Ca^{2+}-bound neurocalcin δ stimulates ANF-RGC. In this situation ANF-RGC response will be very rapid and the cyclic GMP produced will start to inhibit aldosterone synthesis almost immediately. Cyclic GMP synthesized by ANF-RGC affects number of effectors of aldosterone synthesis. They include cyclic GMP-gated channels, cyclic GMP-dependent protein kinases, and cyclic GMP-regulated phosphodiesterases (reviewed in Lohmann et al., 1997; Pfeifer et al., 1999). Several reports indicate that cyclic GMP-driven inhibition of aldosterone synthesis is, at least in part, mediated by cyclic GMP-stimulated phosphodiesterase (PDE 2) that is expressed at high levels in adrenal glomerulosa cells (MacFarland et al., 1991; Côté et al., 1999). This hypothesis needs now experimental validation.

GCAP1 - ANTITHETICAL CALCIUM SENSOR

Since its discovery, GCAP1 has been exclusively regarded as the component of the phototransduction machinery sensing the fall in Ca^{2+} concentration after illumination, transmitting this information to photoreceptor guanylate cyclase ROS-GC and stimulating it to synthesize cyclic GMP at a faster rate (reviewed in Pugh et al., 1997; Koch et al., 2010). With the ensuing raise of Ca^{2+} concentration, Ca^{2+}-bound GCAP1 inhibits ROS-GC activity bringing it to the basal level. This activator/inhibitor mode of GCAP1 operation is well established both *in vivo* and *in vitro*.

Recent work, however, indicated that GCAP1 could target to another sensory membrane guanylate cyclase, an odorant receptor ONE-GC [alternatively termed GC-D (Fülle et al., 1995)] expressed in a subpopulation of olfactory sensory neurons (Duda et al., 2006; Pertzev et al., 2010).

GCAP1 transmits the Ca^{2+}-stimulatory signal to the odorant receptor guanylate cyclase, ONE-GC

Recombinant ONE-GC expressed in COS cell was exposed to increasing Ca^{2+} concentrations and constant, 4 μM, GCAP1 concentration. As a positive control, recombinant ROS-GC1 was treated identically. Both cyclases were expressed in COS to approximately the same level as verified by Western blot (**Figure 6** inset). As expected, based on previous reports (Duda et al., 1996), at or below 10 nM Ca^{2+}, GCAP1 maximally stimulated ROS-GC1 (**Figure 6**: closed circles). The stimulation decreased with increasing free Ca^{2+} concentration and the half-maximal inhibition was at about 100 nM Ca^{2+}. Contrary to that, at about 100 nM Ca^{2+} there was practically no effect on GCAP1-dependent ONE-GC activity (**Figure 6**: open circles). However, with the Ca^{2+} concentrations increasing beyond 100 nM there was a dose-dependent increase in ONE-GC activity. The half-maximal activation of the cyclase occurred at 0.7 μM Ca^{2+} and the maximal activation at about 2.5 μM. At Ca^{2+} concentrations above 2.5 μM there was no statistically significant increase in the cyclase activity. The expression levels of ROS-GC1 and ONE-GC in COS cells was comparable (**Figure 6**: inset). These results essentially confirm the previous published in Duda et al. (2006). The main conclusion is that GCAP1 can function as calcium-dependent regulator of guanylate cyclase activity, but depending on the target it can activate the cyclase in a Ca^{2+}-dependent manner with reverse premise making it an antithetical modulator.

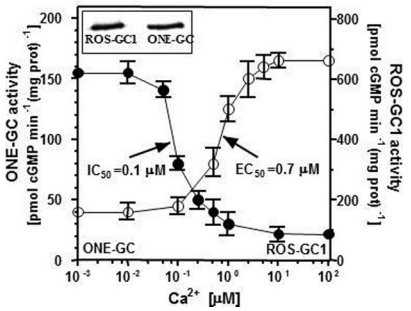

FIGURE 6 | Antithetical Ca^{2+} modulated GCAP1 effect on ONE-GC and ROS-GC1 activities. Membranes of COS cells expressing ONE-GC or ROS-GC1 (control) were individually assayed for guanylate cyclase activity in the presence of 4 μM GCAP1 and indicated concentrations of Ca^{2+}. The experiment was carried out in triplicate and repeated three times. The results shown are shown are average ± SD from these experiments. The IC_{50} and EC_{50} values were determined graphically. **Inset:** Expression levels of ROS-GC1 and ONE-GC in COS cells were monitored by Western blot using antibodies against ROS-GC1 or ONE-GC.

How can GCAP1 exhibit these opposite modulatory effects? To answer this question GCAP1 and ONE-GC mutants were employed.

Several mutations in the GCAP1 gene have been found in patients suffering from autosomal dominant cone-rod dystrophies (Behnen et al., 2010). All these mutations except one are located within the regions coding for EF hands 3 and 4. The $D^{100}E$ mutation is within EF hand 3; it causes perturbation in Ca^{2+} coordination, and leads to a dramatic decrease in affinity for Ca^{2+}. As a consequence, the $D^{100}E$ mutant remains in an active conformation at 10 μM Ca^{2+}. The activation is half-maximal at 20 μM Ca^{2+} and the cyclase activity returns to the basal level only at approx. 60 μM free Ca^{2+} (Behnen et al., 2010; Dell'Orco et al., 2010). This mutant was tested for its effect on ONE-GC activity in the presence of two Ca^{2+} concentrations, 10 nM and 10 μM. For comparison, in parallel experiment, the effect of the $D^{100}E$ mutant on ROS-GC1 activity at the same Ca^{2+} concentrations was determined. The results are shown in **Figure 7**. The mutant stimulated ROS-GC1 activity at both tested Ca^{2+} concentrations. It, however, had no effect on ONE-GC activity at 10 nM Ca^{2+} but stimulated its activity at 10 μM Ca^{2+}. Thus, dysfunctional EF hand 3 does not have any effect on Ca^{2+}-dependent GCAP1 stimulation of ONE-GC. Because the third EF hand has the highest

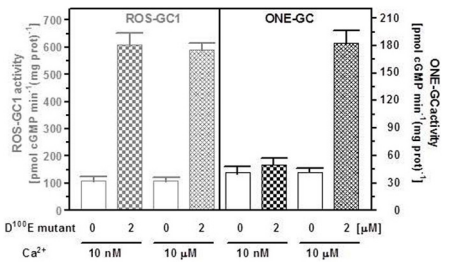

FIGURE 7 | The $D^{100}E$ GCAP1 mutant stimulates both ONE-GC and ROS-GC1 at high Ca^{2+} concentration but only ROS-GC1 in the absence of Ca^{2+}. The membrane fraction of COS cells expressing ONE-GC or ROS-GC1 (control) were assayed for guanylate cyclase activity in the presence (2 μM) or absence of $D^{100}E$ GCAP1 mutant and 1 mM EGTA (10 μM Ca^{2+}) or 10 μM Ca^{2+}. The experiment was done in triplicate and repeated two times for reproducibility. The results shown are average ± SD from these experiments.

affinity for Ca^{2+} (Lim et al., 2009) these results indicated that low affinity Ca^{2+} binding to GCAP1 is sufficient for ONE-GC stimulation. These results further showed that the $D^{100}E$ mutation in GCAP1 leads to a protein conformation that can act as a constitutive activator with Ca^{2+}-sensing properties that are significantly shifted to higher free Ca^{2+}. One can deduce from these results that the interaction sites of GCAP1 in ROS-GC1 and ONE-GC are different, i.e., are not located in corresponding homologous regions. This hypothesis was tested by creating a mutant of ONE-GC with a point mutation in a region that is conserved among sensory guanylate cyclases and that is critical for GCAP1-dependent regulation of ROS-GC1.

GCAP1 signaling modes in its Ca^{2+}-free and Ca^{2+}-bound states are different

The first ROS-GC1 gene mutation linked with visual disorder was the $F^{514}S$ mutation identified in cases of Leber's congenital amaurosis type 1 (LCA1) (Perrault et al., 1996). Studies aimed at explaining the molecular basis of LCA1 demonstrated that this mutation totally disables GCAP1 modulation of ROS-GC1 activity (Duda et al., 1999). Because phenylalanine in this position is conserved in all membrane guanylate cyclases, the obvious question to ask was: would a similar mutation in ONE-GC disable GCAP1-mediated Ca^{2+} signaling of its activity? ONE-GC $F^{585}S$ mutant (corresponding to the $F^{514}S$ in ROS-GC1) was constructed and its activity was determined in the presence of 10 μM Ca^{2+} and increasing concentrations of GCAP1 (**Figure 8A**: closed circles). The results show that contrary to ROS-GC1 and its $F^{514}S$ mutant (**Figure 8A**: open and closed triangles, respectively; the activity was measured in the absence of Ca^{2+}), the F→S mutation in ONE-GC does not affect the Ca^{2+}-GCAP1-dependent activation of the cyclase (**Figure 8A**: compare the profiles with closed and open circles). The Hill's coefficients calculated for the GCAP1 stimulation of wt ONE-GC and of its $F^{585}S$ mutant were virtually identical, 1.83 ± 0.3. The mutation also does not affect the Ca^{2+} sensitivity of the mutant (**Figure 8B**: compare the closed and

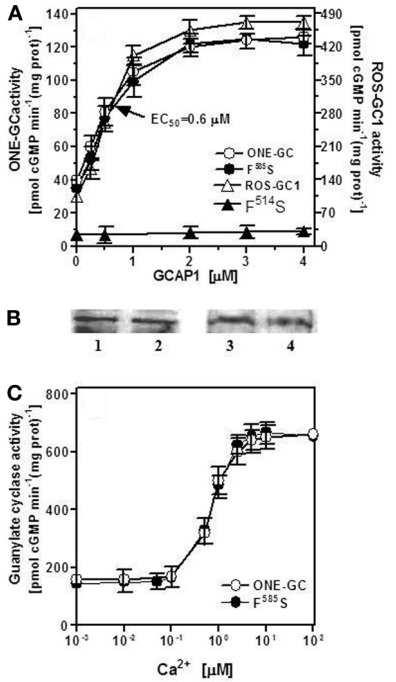

FIGURE 8 | $F^{585}S$ mutation does not affect Ca^{2+}-GCAP1 signaling of ONE-GC activity. (A) Membranes of COS cells expressing ONE-GC or its $F^{585}S$ mutant were assayed for guanylate cyclase activity in the presence of increasing concentrations of GCAP1 and constant 10 μM Ca^{2+}; Membranes of COS cells expressing ROS-GC1 or its $F^{514}S$ mutant were analyzed for GCAP1 effect in the absence of Ca^{2+} (1 mM EGTA was present in the assay mixture). **(B)** Membranes of COS cells expressing ONE-GC and its $F^{585}S$ mutant or ROS-GC1 and its $F^{514}S$ mutant were analyzed by Western blot using antibodies against ROS-GC1 and ONE-GC. Approx 20 μg of membrane protein was applied on each lane of the gel. Lane 1, ONE-GC; Lane 2, ONE-GC $F^{585}S$; Lane 3, ROS-GC1; Lane 4, ROS-GC1 $F^{514}S$. **(C)** Membranes of COS cells expressing ONE-GC or its $F^{585}S$ mutant were assayed for guanylate cyclase activity in the presence of increasing concentrations of Ca^{2+} and constant, 4 μM GCAP1. The experiments were done in triplicate and repeated for reproducibility with different preparations of transfected COS cells. The results presented (mean ± SD) are from one representative experiment.

open circles). It is, therefore, concluded that Ca^{2+}-free and Ca^{2+}-bound GCAP1 exert different signals for activation of membrane guanylate cyclase. They probably do so by acting on different target regions, since the mutation $F^{514}S$ is located in a previously identified interaction and/or regulatory region of ROS-GC1 (Lange et al., 1999). Finally, while the $F^{514}S$ mutation in ROS-GC1 results in blindness at birth or soon thereafter (Perrault et al., 1996), the corresponding $F^{585}S$ mutation in ONE-GC would not result in anosmia.

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