EMERGING MECHANISMS IN PURINERGIC SIGNALING: FROM CELL BIOLOGY TO THERAPEUTIC PERSPECTIVES

EDITED BY: Rosa Gomez-Villafuertes and Elena Adinolfi
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EMERGING MECHANISMS IN PURINERGIC SIGNALING: FROM CELL BIOLOGY TO THERAPEUTIC PERSPECTIVES

Topic Editors:
Rosa Gomez-Villafuertes, Complutense University of Madrid, Spain
Elena Adinolfi, University of Ferrara, Italy

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EDITORIAL
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Editorial: Emerging Mechanisms in Purinergic Signaling: From Cell Biology to Therapeutic Perspectives

Rosa Gomez-Villafuertes[1,2,3]* *and Elena Adinolfi*[4]*

[1] Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain, [2] Instituto Universitario de Investigación en Neuroquímica, Universidad Complutense de Madrid, Madrid, Spain, [3] Instituto de Investigación Sanitaria del Hospital Clínico San Carlos, Madrid, Spain, [4] Section of Experimental Medicine, Department of Medical Sciences, University of Ferrara, Ferrara, Italy

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Editorial on the Research Topic

Emerging Mechanisms in Purinergic Signaling: From Cell Biology to Therapeutic Perspectives

Purinergic signaling or purinome is the common name for a complex ensemble of receptors, extracellular enzymes, and transporters interacting with extracellular ATP and its degradation products ADP, AMP, and adenosine. It was thanks to the early discovery of professor Burnstock that ATP, previously tough to exert its function manly intracellularly, could also activate extracellular signals and to his strong support to the existence of a non-adrenergic non-cholinergic neurotransmission that purinergic signaling took its first steps. Subsequently, many studies proved that the purinome is involved not only in neurotransmission but also in the activation of the immune response, carcinogenesis and the etiopathology of several conditions including but not limited to neuropathic and inflammatory pain, neurological disorders, cardiovascular, infectious, skeletal, reproductive, and immune system diseases, alterations of sense organs, airways, skin, muscles, gut, kidney and urinary tract. The purinome includes the P2X ATP-gated ion channels, metabotropic receptors for ATP (P2Y) and Adenosine (ADORA) but also ectonucleotidases such as CD39 and CD73 that are responsible for the hydrolysis of ATP to its derivatives ADP, AMP and adenosine. The intracellular pathways activated by this plethora of receptors include classical G-proteins coupled c-AMP and inositol triphosphate pathways, but also kinases and nuclear factors among which MAPK, Akt, PI3K, NF-kB, HIF-1α and NFAT. From a pharmacological point of view, several potent and selective agonists/antagonists, allosteric modulators, and blocking antibodies targeting most purinergic receptors and ectonucleotidases are available. Some of these compounds are currently used in therapy such as the antithrombotics targeting $P2Y_{12}$ receptor, and multiple clinical trials are in progress to explore purinergic agents for the treatment of different neurological, inflammatory, and oncologic diseases among others. Nevertheless, a further research effort is required to better identify the right drugs or agonist/antagonist combinations and diseases to be targeted with purinome-centered therapies. This Research Topic aimed to gather new data and opinions on physio-pathological roles played by the purinome components.

Research articles in this topic include a brief report by Pegoraro et al. that investigates the effect of P2X7 receptor expression on HHV-6A infection, showing that P2X7 antagonism decreases viral load. Moreover, the authors demonstrate that P2X7 489C>T polymorphism correlates with HHV-6A infection in women affected with idiopathic infertility, a condition previously shown to correlate with HHV-6A infection. These data point to the P2X7 receptor as a potential therapeutic target to prevent HHV-6 infection and associated infertility.

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Salvatore Salomone, University of Catania, Italy

***Correspondence:**
Rosa Gomez-Villafuertes
marosa@ucm.es
Elena Adinolfi
elena.adinolfi@unife.it

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Previous studies suggest that both ATP and adenosine consistently reduce the sinoatrial node spontaneous activity leading to negative cardiac chronotropy. Here, Bragança et al. demonstrate that activation of P2X4 ionotropic receptors plays a major role in decreasing the spontaneous activity of the sinoatrial node while partially offsetting the negative inotropic effect of the nucleotide in paced rat ventricles. These results strongly suggest that P2X4 agonists have the potential to became novel well-tolerated heart-rate lowering drugs with promising benefits in patients with deteriorated ventricular function.

In the field of neuroprotection, Alves et al. analyze the expression of P2Y receptors in the cortex following status epilepticus and determine the impact $P2Y_1$ modulation on cortical damage using a unilateral mouse model of intra-amygdala kainic acid-induced status epilepticus. This study extends previous data and confirms anticonvulsive and neuroprotective properties of $P2Y_1$ antagonism during status epilepticus, suggesting $P2Y_1$-based treatment as possible new therapy for drug-resistant status epilepticus.

The ecto-5'-nucleotidase CD73 plays an important role in regulating vascular permeability and immune cell function. In this regard, Caiazzo et al. evaluate the effect of CD73 inhibition in the development of inflammation in the carrageenan-induced pleurisy model. This study demonstrates that inhibition of CD73 exacerbates the early phase of carrageenan-induced pleurisy by controlling pleural effusion and polymorphonuclear migration *in vivo* and *ex vivo*. On this basis, the authors suggest that CD73 might represent a valid biomarker for pleural effusion and a potential target for novel therapeutic interventions.

Another study included in the topic and covering lung inflammation is that of Santana et al., which investigates the role of the $P2Y_{12}$ receptor in silicosis, demonstrating that inhibition of $P2Y_{12}$ signaling with clopidogrel prevents silica-induced changes in lung function, and significantly reduces lung inflammation, fibrosis, as well as cytokine and nitrite production, thus preventing lung functional alterations and mortality.

Overviews by purinome experts are also present in this Research Topic including the article by Stokes et al. that summarizes the current evidence on the physiological roles of P2X receptors and discuss whether the use of pharmacological agents enhancing P2X receptor activity would offer a therapeutic benefit. Based on the advances in structural information and continued progress in allosteric binding pocket identification, plus access to the relevant animal models of disease, the authors convincingly suggest that positive modulation of P2X receptors will become a new fruitful area of research.

Corciulo and Cronstein give an overview of the purinergic system in the joint describing the expression and function of purinome components in the synovia, cartilage, ligament, tendon, and bone and highlighting the therapeutic perspective of targeting purinergic signaling in this anatomical area.

Similarly, Khalafalla et al. summarize the role of purinergic receptors in salivary gland function and dysfunction, analyzing their potential as therapeutic targets to promote saliva flow, prevent salivary gland inflammation, and enhance tissue regeneration.

In addition, Wei et al. review recent studies that support the role of Piezo1 channel as an intrinsic mechanosensor to trigger ATP release and subsequent purinergic receptor activation in several types of mechanosensitive cell types such as urothelial cells, endothelial cells, red blood cells, and mesenchymal stem cells.

In conclusion, we believe that the articles presented in this Research Topic cover multiple aspects of the ongoing research in the purinergic field and show the difficult task of fully-understanding the complexity of this ubiquitous signaling cascade.

Finally, we would like to dedicate the issue to the memory of Professor Geoffrey Burnstock, who recently passed away. He will always be an example of genius and passion for us and will be much missed by the scientific community. May he rest in peace and continue challenging dogmas wherever he is.

AUTHOR CONTRIBUTIONS

RG-V and EA have equally contributed to the work, and approved it for publication.

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ORIGINAL RESEARCH
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The Ecto-5'-Nucleotidase/CD73 Inhibitor, α,β-Methylene Adenosine 5'-Diphosphate, Exacerbates Carrageenan-Induced Pleurisy in Rat

*Elisabetta Caiazzo[1], Silvana Morello[2], Rosa Carnuccio[1], Armando Ialenti[1] and Carla Cicala[1]**

[1] Department of Pharmacy, School of Medicine, University of Naples Federico II, Via Domenico Montesano 49, Naples, Italy,
[2] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, Fisciano, SA, Italy

The ecto-5'-nucleotidase (ecto-5'NT/CD73) represents a crucial enzyme for endogenous adenosine generation. Several findings have shown that CD73 plays an important role in regulating vascular permeability and immune cell function. Adenosine 5'-(α,β-methylene)diphosphate (APCP) is a CD73 inhibitor, widely used as pharmacological tool to investigate the role of CD73/adenosine pathway in several *in vitro* and *in vivo* models, although it has been also shown to inhibit other ectoenzymes involved in adenosinergic pathway. Here, we evaluated the effect of APCP in the development of inflammation in carrageenan-induced pleurisy model. We found that treatment with APCP (400 μg/rat) significantly increased cell accumulation, exudate formation, and pro-inflammatory cytokine content into the pleural cavity in the acute phase (4 h) of inflammation, with no differences in the sub-acute phase (72 h) except for the regulation of monocyte chemotactic protein-1 levels. In addition, cells collected by pleural lavage fluids of APCP-treated rats, 4 h following carrageenan injection, showed increased ability to migrate *in vitro*, both in presence and in absence of N-formyl-L-methionyl-L-leucyl-L-phenylalanine as chemotactic stimulus, compared to cells obtained by control rats. Our results demonstrate that APCP exacerbates the early phase of carrageenan-induced pleurisy by controlling pleural effusion and polymorphonuclear migration *in vivo* and *ex vivo*. This effect is likely dependent upon CD73 inhibition, although an inhibitory effect of other ectoenzymes cannot be ruled out.

Keywords: adenosine, CD73, ecto-5'-nucleotidase, inflammation, pleurisy, rat

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Reviewed by:
Luca Antonioli, University of Pisa, Italy
Ronald Sluyter, University of Wollongong, Australia

****Correspondence:***
Carla Cicala
cicala@unina.it

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INTRODUCTION

Ecto-5'-nucleotidase (ecto-5'NT/CD73), the key enzyme in leading to adenosine accumulation, has been localized on barrier cell types, such as endothelial cells, and participates in the control of barrier permeability. Thompson and coworkers (2004) by performing experiments on genetically modified mice, lacking CD73, were the first to demonstrate the important role for this ecto-enzyme in the control of the vascular leakage following hypoxia, a common feature of inflamed tissues. This finding was in agreement with previous experimental work demonstrating that following hypoxia the increased CD73 activity and extracellular adenosine accumulation represent

a protection preserving intestinal epithelium from vascular leakage. On the other hand, administration to mice of the CD73 inhibitor α,β-methylene adenosine 5'-diphosphate (APCP) significantly increased the permeability of intestinal epithelium (Synnestvedt et al., 2002; Ledoux et al., 2003). Although CD73 has been shown to play an important role in the development of pulmonary inflammation and protection against lung injury induced by artificial ventilation in mice (Eckle et al., 2007), less is known on changes of its expression and activity on tissues and cells following inflammation. Indeed, in an inflammatory environment, recruited and stromal cells expressing CD73 are critical producers of adenosine that, in turn, by engaging its receptors on adjacent cells, exerts an immunomodulatory effect (Antonioli et al., 2013).

Pleurisy is characterized by inflammation of the pleura, the tissue composed of mesothelial cells, lining the lung and regulating pleural cavity homeostasis. Carrageenan-induced rat pleurisy is a model characterized by an early (4 h) and late (72 h) phase of inflammation, and it is a useful model to study pleural effusion, resulting in extravasation and cell accumulation into the pleural space (Murai et al., 2003) that may represent a severe complication of pulmonary and extra pulmonary diseases.

Aim of the present study was to analyze the role of CD73 in pleural effusion, in rat. We found that treatment with the CD73 inhibitor, APCP, influences the accumulation of leukocytes and cytokine production within pleural cavity. These effects were associated with worsened lung injury and enhanced ability of pleural leukocytes to migrate. All together, our data suggest that CD73 represents an endogenous modulator of pleural effusion during the early phase of inflammation.

MATERIALS AND METHODS

Animals

All experiments were performed on male Wistar rats (220–260 g; Charles River, Calco, Italy). The animals were maintained at a room temperature of 22 ± 2° on a 12 h/12 h light/dark cycle and were housed in a specific pathogen-free environment and fed standard rodent chow and water *ad libitum*. All procedures were performed according to the Italian (DL 26/2014) and European (n.63/2010/UE) regulations on the protection of animals used for experimental and other scientific purposes and were approved by Italian Ministry of Health (Number 1039/2016).

Carrageenan-Induced Pleurisy

Rats were anesthetized with 4% enflurane mixed with 0.5 L/min O_2 and 0.5 L/min N_2O, and a skin incision at the level of the left sixth intercostal space was performed. The underlying muscle was dissected, and 0.2 ml of λ-carrageenan type IV (1% w/v; Sigma-Aldrich, Milan, Italy) was injected into the pleural cavity. The CD73 inhibitor, APCP (400 μg/rat; Tocris Bioscience, Bristol, UK), or an equal volume of the vehicle (distilled water), was injected into the pleural cavity immediately before carrageenan injection. The skin was then sutured, and animals were returned to their cages and allowed to have food and water *ad libitum*; 4 and 72 h following pleurisy induction, rats were sacrificed by CO_2 inhalation. The chest was carefully opened, and the pleural cavity was washed with 2 ml of sterile saline containing 10 U/ml heparin (Sigma-Aldrich, Milan Italy). Any lavage fluid with blood contamination was rejected. The volume of pleural lavage fluid collected from each animal was measured; then, fluids were centrifuged at 180 × g for 10 min and the pellet suspended in phosphate-buffered saline (PBS). Cells were counted with TC20™ Automated Cell Counter (Bio-Rad, Italy). Samples of supernatants and cell pellets were then frozen at −80°C to be successively analyzed for cytokine content and AMPase activity, whereas differential cell count was performed in smears by May-Grunwald-Giemsa staining (Carlo Erba, Italy). Lung samples were harvested from each rat and immediately frozen at −80°C or fixed in formaldehyde solution (4% v/v, in distilled water) for 1 week at room temperature and successively utilized for further analyses.

Morphological Analysis

Morphological analysis was performed on pulmonary tissue samples from vehicle- and APCP-treated rats. Lung biopsies harvested 4 or 72 h following carrageenan injection and fixed in formaldehyde, as described above, were dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (7-μm thickness) were then deparaffinized with xylene and stained with hematoxylin and eosin (Kaltek, Padova, Italy). A minimum of five sections *per* animal was analyzed under direct light microscopy (original magnification X 20) and photographed by a Leica DFC320 video camera (Leica, Milan, Italy) connected to a Leica DM RB microscope by using the Leica Application Suite software V 4.1.0.

AMPase Activity

AMPase activity, evaluated as previously described (Caiazzo et al., 2016), was assessed in cells, lung samples, and cell-free pleural lavage fluids collected from rats 4 or 72 h following pleurisy induction, by colorimetric measurement of the inorganic phosphate (P*i*) released following incubation with the substrate, as described by Nedeljkovic et al. (2006). Cell pellets were suspended in 50 μl of ice-cold lysis buffer [4-[2-hydroxyethyl]-1-piperazine ethane sulfonic acid (HEPES), 200 mM; NaCl, 400 mM; dithiothreitol (DTT), 1 mM; IGEPAL, 1%; glycerol, 20% (Carlo Erba, Italy), plus the protease inhibitor cocktail (Roche, Italy)] and maintained in constant agitation for 45 min at 4°C. Lung tissue was transferred into a tube preloaded with one (6.35 mm) diameter zirconium oxide coated ceramic grinding sphere in ice-cold lysis buffer [Tris-HCl, 50 mM pH 7.5; NaCl, 150 mM; sodium orthovanadate, 1 mM; β glycerophosphate, 20 mM; ethylenediaminetetraacetic acid (EDTA), 2 mM; phenylmethylsulfonyl fluoride (PMSF), 1 mM; leupeptin, 5 μg/ml; aprotinin, 5 μg/ml; pepstatin, 5 μg/ml; ICN Pharmaceuticals, Italy] and put into a FastPrep®-24 homogenizer (MP Biomedicals, Santa Ana, California, USA) for lysis. Cell lysates and lung homogenates were then centrifuged for 15 min at 8,000 × g at 4°C, and the supernatant (protein extract) was collected and stored at −80°C until analysis. To initiate the enzymatic reaction, samples (50 μg of proteins) were incubated with 200 μl of medium containing $MgCl_2$ (10 mM), NaCl (120 mM),

KCl (5 mM), glucose (60 mM), Tris-HCl (50 mM), and pH 7.4. After 10 min, AMP (2 mM) was added as substrate and samples kept at 37°C for 40 min. The reaction was then stopped by the addition of trichloroacetic acid (final concentration 5% w/v). Following sample centrifugation at 500 × g for 10 min, at 37°C, the release of P*i* was measured using Malachite Green Phosphate Assay Kit (ScienCell, Research Laboratories, Carlsbad, USA) and KH_2PO_4 as standard (Chan et al., 1986). To have the net value of P*i* produced following enzymatic reaction, aspecific P*i* released in the absence of AMP in each sample was evaluated and the value obtained was subtracted from the value obtained following incubation with AMP. Protein concentration was measured by Bradford assay, and results were expressed as P*i* released (pmol/min/μg protein). AMPase activity was also assessed in cell-free pleural lavage fluids collected from rats 4 and 72 h following pleurisy induction by performing the same procedure described above.

Cytokine Measurement

In the pleural lavage fluids collected 4 and 72 h following carrageenan injection, levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) were evaluated by a colorimetric commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions and expressed as pg *per* ml.

Chemotaxis Assay

Chemotaxis was evaluated on cells obtained from pleural lavage fluids collected 4 h after carrageenan injection in a 48-well modified Boyden chamber (AP48, Neuro Probe, USA). Briefly, 2.50×10^5 cells in Roswell Park Memorial Institute (RPMI-1640) medium containing 0.1% bovine serum albumin (BSA) (50 μl) were placed on top of the polycarbonate filter (8-μm pore size, Neuro Probe, USA) whereas 25 μl of chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 1 ng/ml in RPMI-1640 medium containing 0.1% BSA) were added to the wells on the bottom. Spontaneous migration was determined using RPMI-1640 medium without fMLP. Each condition was set up in triplicate. Chambers were incubated for 90 min at 37°C in humidified air containing 5% CO_2. Following incubation, the chamber was disassembled and the filter was carefully removed and washed with sterile PBS. Cells that failed to migrate through the filter were wiped off the top surface of the filter. Migrated cells on the filter were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, ON, Canada), and migration was quantified as the total pixel count of DAPI-stained nuclei under the fluorescence microscope using Fiji software. Migration index of cells obtained from pleural lavage fluids, harvested from vehicle- and APCP-treated rats, was expressed as ratio between the number of migrated cells in presence of fMLP and the number of migrated cells in the absence of fMLP.

Flow Cytometry

Pleural lavage fluids recovered from the pleural cavity were washed twice in saline, and the resulting suspensions were pelleted by centrifugation at 180 × g for 10 min at 4°C. To block non-specific Fc-mediated interactions, cell samples were pre-incubated with an FcR blocking reagent (BD Biosciences) on ice for 10 min and then incubated with the following antibodies against: CD11b/c-PerCP/Cy5.5 (clone OX-42, BioLegend), RP-1-PE (BD Pharmingen), CD68-FITC (clone ED1, GeneTex), and CD73-Alexa Fluor 647 (Bioss Antibodies) for 20 min in the dark. Data were acquired with a FACSCalibur flow cytometer and analyzed with CellQuest software (BD FACSCalibur, Milan, Italy).

Statistical Analysis

All data are presented as mean ± standard error (SE); statistical analysis was performed on raw data by two tailed Student's t-test for unpaired data or by one-way ANOVA followed by Bonferroni *post-hoc* test as appropriate. A p value < 0.05 was considered statistically significant.

RESULTS

APCP Treatment Increases Pleural Effusion and Cell Infiltration

Treatment of rats with CD73 inhibitor, APCP (400 μg/rat), significantly increased cell accumulation into the pleural cavity 4 h following carrageenan injection compared to vehicle (**Figure 1A**), while the leukocyte number into the pleural lavage fluids collected at 72 h after carrageenan injection was similar in APCP- and vehicle-treated rats (**Figure 1A**). The volume of exudate collected by pleural lavage 4 h following carrageenan injection significantly increased in APCP-treated rats compared with control (vehicle-treated) rats (**Figure 1B**). There was no difference in the volume of exudate produced at 72 h after carrageenan injection between APCP- and vehicle-treated rats (**Figure 1B**).

APCP Treatment Increases Lung Damage

Morphological analysis of inflamed lung sections showed cell infiltration into bronchial and perivascular space as well as lung injury (**Figure 2**). The increased inflammatory cell infiltration in APCP-treated rats was associated with damaged lung architecture 4 and 72 h following carrageenan-induced pleurisy (**Figure 2**).

APCP Treatment Is Associated With Reduced AMPase Activity

In APCP-treated rats, the AMPase activity in cell lysates, in lung homogenates, and in cell-free pleural lavage fluids 4 h following carrageenan injection was significantly reduced compared with vehicle, but not at 72 h (**Figures 3A**, **B**, and **C**, respectively). It is worth noting that the AMPase activity in cell lysates at 72 h was higher than at 4 h (**Figure 3A**) and in cell-free pleural lavage fluids was lower at 72 h than at 4 h (**Figure 3C**). In lung homogenates, there was no difference in AMPase activity between 4 and 72 h (**Figure 3B**).

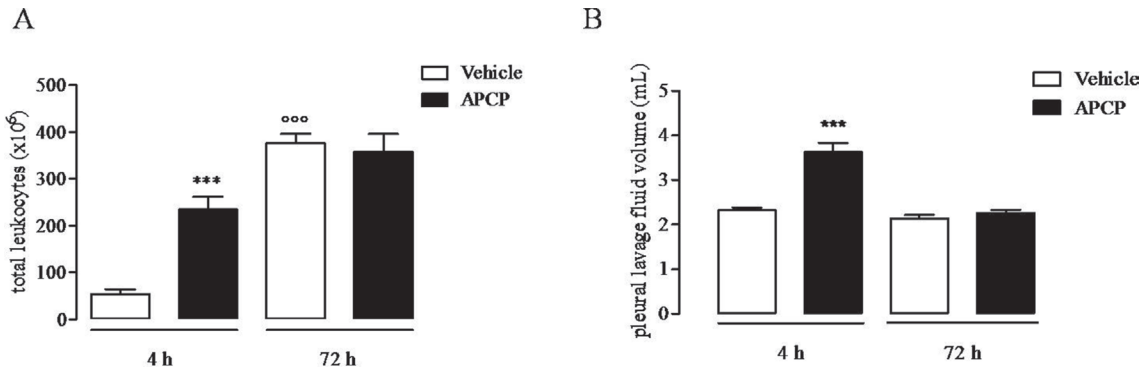

FIGURE 1 | Effect of APCP on leukocyte infiltration and pleural lavage fluid volume 4 and 72 h following carrageenan-induced rat pleurisy. **(A)** Total leukocytes. **(B)** Pleural lavage fluid volume. Data are expressed as mean ± SE, N = 10; **(A)** ***p < 0.001 and °°°p < 0.001 *versus* vehicle 4 h. **(B)** ***p < 0.001 *versus* vehicle 4 h. Two-tailed Student's t-test.

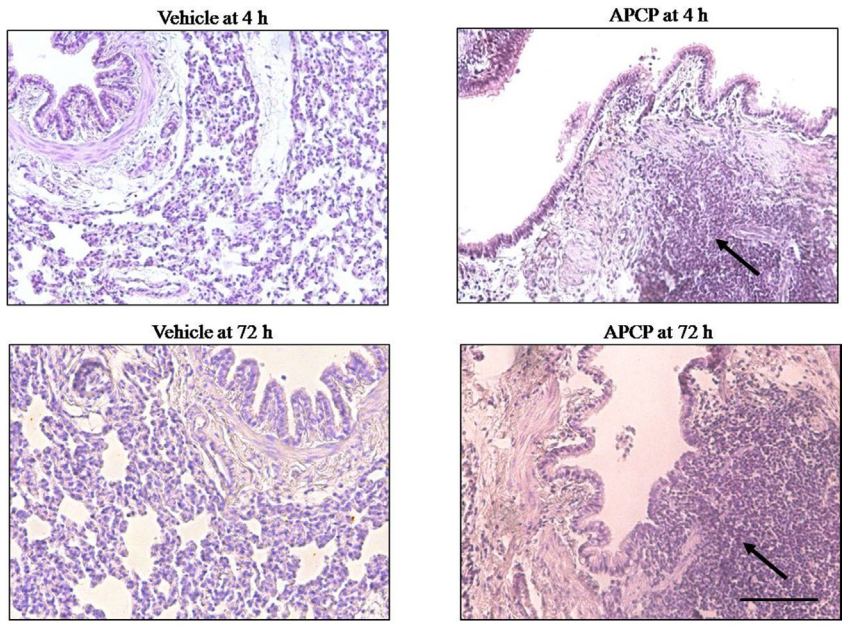

FIGURE 2 | Effect of APCP on lung injury 4 and 72 h following carrageenan-induced pleurisy. Representative morphological analysis of hematoxylin and eosin (H&E)-stained lung sections from vehicle group (4 and 72 h) and APCP group (4 and 72 h). Black arrows indicate points of intense inflammatory cell infiltration. Original magnification X 20. Scale bar = 100 μm.

APCP Treatment Increases Cytokine Production in Pleural Fluids

Following treatment with APCP, there was a significant increase in TNF-α, IL-6, IL-1β, and MCP-1 levels evaluated in cell-free pleural lavage fluids collected 4 h following pleurisy induction (**Figures 4A**, **B**, **C**, and **D**, respectively). In contrast, TNF-α, IL-6, and IL-1β levels in pleural lavage fluids collected at 72 h were similar in APCP- and vehicle-treated rats (**Figures 4A**, **B**, and **C**, respectively), while MCP-1 levels were still significantly increased in APCP-treated rats compared with control (vehicle-treated) at 72 h (**Figure 4D**).

APCP Treatment Increases PMN Migration Index

Cells obtained from pleural lavage fluids collected from APCP-treated group of rats 4 h after carrageenan injection showed increased ability to migrate *in vitro* both in presence and in absence of a chemotactic stimulus (fMLP), compared to cells from vehicle groups, as showed in the representative images of **Figure 5A**. Accordingly, the migration index was significantly increased in samples from APCP-treated rats compared with control (vehicle-treated rats) in presence of fMLP (**Figure 5B**).

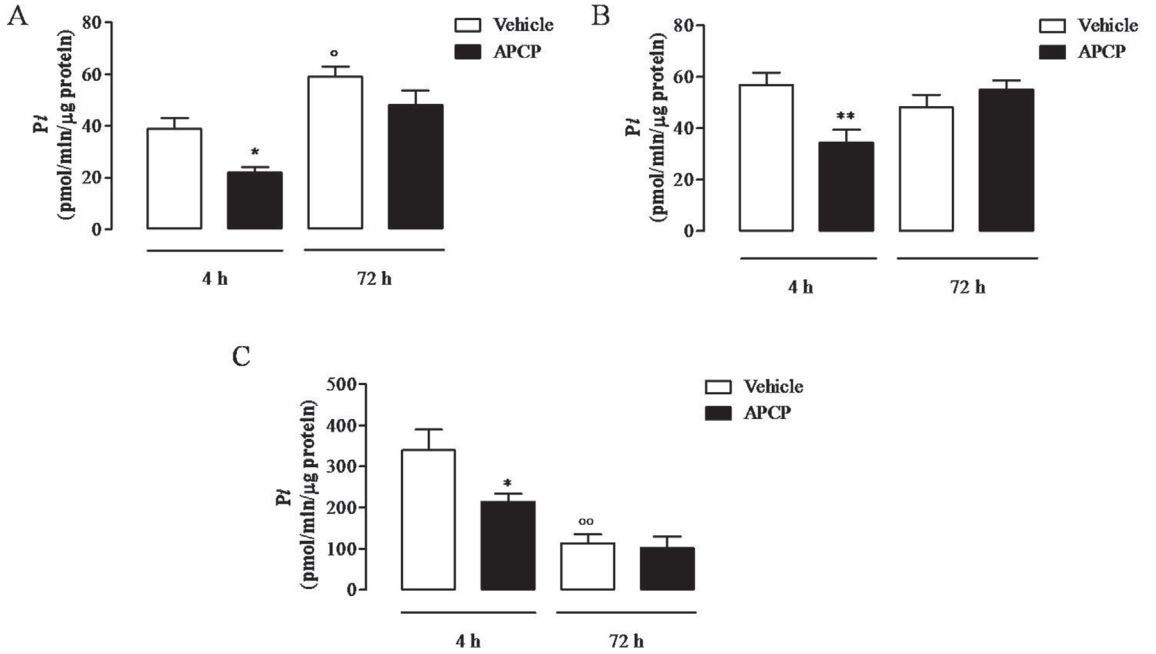

FIGURE 3 | AMPase activity in cells **(A)**, lungs **(B)**, and pleural lavage fluids **(C)** collected 4 and 72 h following carrageenan-induced pleurisy. Data are expressed as mean ± SE, N = 6; **(A)** *p < 0.05 and °p < 0.05 *versus* vehicle 4 h. **(B)** **p < 0.01 *versus* vehicle 4 h. **(C)** *p < 0.05 and °°p < 0.01 *versus* vehicle 4 h. Bonferroni's multiple comparison test.

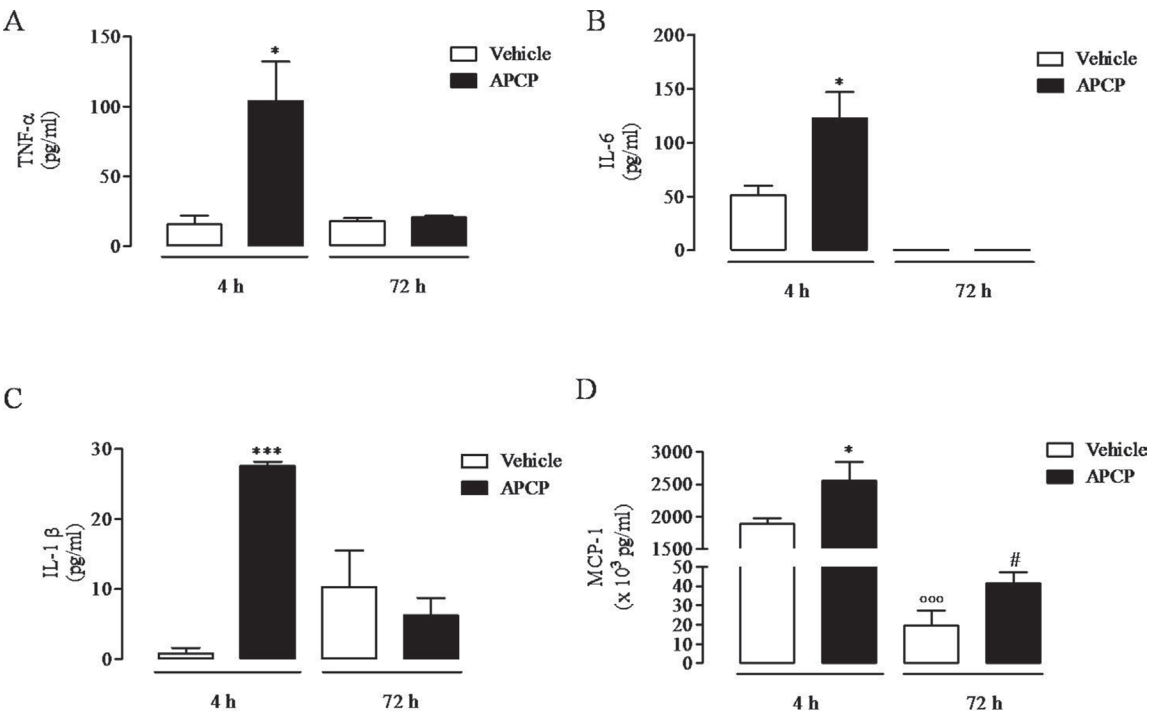

FIGURE 4 | Effect of APCP on levels of TNF-α **(A)**, IL-6 **(B)**, IL-1β **(C)**, and MCP-1 **(D)** in pleural lavage fluid recovered from the pleural cavity 4 and 72 h following carrageenan-induced rat pleurisy. Data are expressed as mean ± SE, N = 6; **(A)** *p< 0.05 *versus* vehicle 4 h. **(B)** *p< 0.05 *versus* vehicle 4 h. **(C)** ***p < 0.001 *versus* vehicle 4 h. **(D)** *p < 0.05 and °°°p < 0.001 *versus* vehicle 4 h; #p < 0.05 *versus* vehicle 72 h. Two-tailed Student's t-test.

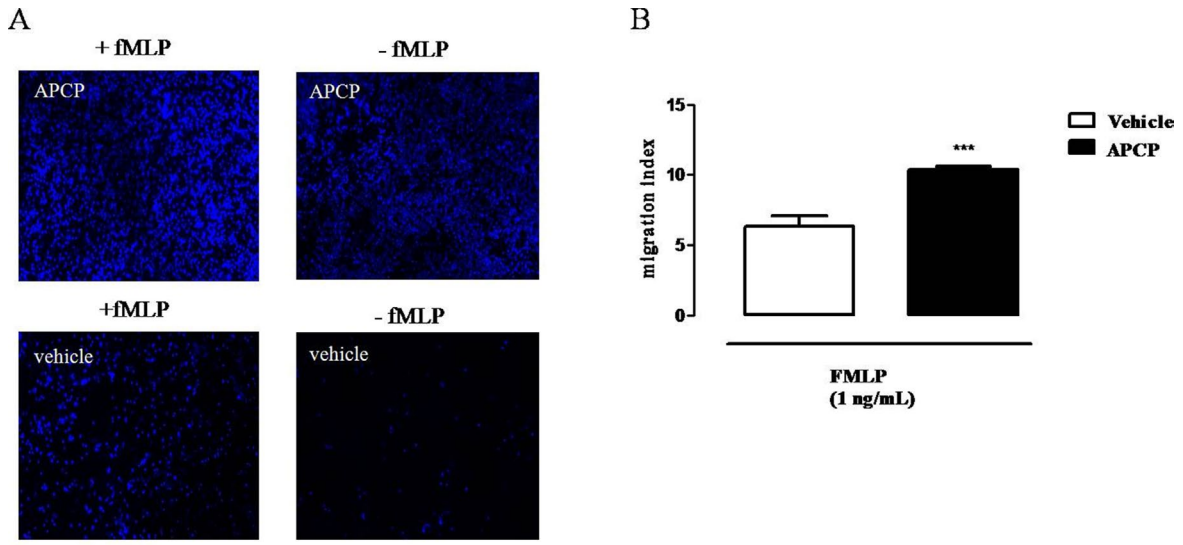

FIGURE 5 | Effect of APCP on chemotaxis of leukocytes collected 4 h after carrageenan injection. **(A)** Representative immunofluorescence showing DAPI-stained nuclei from vehicle and APCP-treated group; cell chemotaxis was evaluated in presence and in absence of fMLP (see text for details). Original magnification X 10. **(B)** Histogram representing the results of migration index expressed as ratio between the number of migrated cells in presence of fMLP and the number of migrated cells without fMLP. Cells were collected from pleural lavage fluids harvested from vehicle- and APCP-treated rats. Data are expressed as mean ± SE, N = 8. ***p < 0.001 *versus* vehicle. Two-tailed Student's t-test.

Leukocyte Populations into the Pleural Cavity

Differential cell count of leukocytes migrated into the pleural cavity showed that PMN neutrophils dominated the early phase (4 h) of the reaction and were replaced by monocytes at 72 h (data not shown). To assay neutrophil and monocyte cell subsets and the expression of CD73, cells collected from pleural lavage fluids at 4 and 72 h following pleurisy induction were analyzed by flow cytometry. Cells collected at 4 hours were mostly neutrophils (CD11b/c$^+$RP-1$^+$ cells) with no difference from APCP- and vehicle-treated group (**Figure 6A**). Moreover, the percentage of CD73$^+$ cells among them (CD11b/c$^+$RP-1$^+$ CD73$^+$) was very low in both groups (**Figure 6A**). Flow cytometric analysis of cells collected at 72 h showed that the main population within leucocytes were represented by monocytes/macrophages (CD11b/c$^+$CD68$^+$ cells) that were significantly reduced in pleural fluids harvested from APCP-treated rats compared with those from vehicle-treated rats (**Figure 6B**). In both vehicle- and APCP-treated groups, these cells were positive to CD73 (CD11b/c$^+$CD68$^+$CD73$^+$cells) (**Figure 6B**). It is worth noting that another subset of cells, CD11b/c$^+$CD68$^-$, represented about 40% of cells harvested at 72 h.

DISCUSSION

There is much evidence for the role of CD73 in maintenance the integrity of epithelial barrier (Narravula et al., 2000; Salmi and Jalkanen, 2005; Bowser and Broaddus, 2016). Early papers investigating the involvement of CD73 in physiopathological conditions found that this enzyme was an innate protection in lung injury (Eckle et al., 2007). Pleural mesothelium is a monolayer of mesothelial cells lining the pleural cavity, presenting intermediate features between epithelium and endothelium; mesothelial cells participate to the pleural space homeostasis and represent the primary cells that initiate the response to an injury (Mutsaers, 2004; Batra and Antony, 2015).

Injection of carrageenan into the rat pleural cavity elicits an inflammatory response characterized by pleural effusion that dominates the early phase (4 h), characterized by cell recruitment, mostly PMNs, cytokine production, and tissue morphological changes. The model may resemble human pleural effusion, a severe complication often associated to pulmonary and extra pulmonary pathologies (Vinegar et al., 1982; De Brito, 1989; Murai et al., 2003; Lansley et al., 2017). Here, we have evaluated the effect of APCP, a CD73 inhibitor, in the inflammatory response triggered by carrageenan, in the model of rat pleurisy.

Following rat treatment with APCP, we found increased pleural effusion and total cell accumulation into the pleural cavity, in response to carrageenan injection, 4 h thereafter. At the same time point, the increased cell infiltration was paralleled by increased cytokine levels (IL-6, TNF alpha, IL-1 beta, MCP-1) into the pleural cavity. Conversely, in the pleural lavage fluids collected 72 h following injection of carrageenan, cytokine levels were almost absent in agreement with evidence of their early involvement in this model of inflammation (Goodman et al., 1993; Murai et al., 2003). Furthermore, at 72 h, there was no difference in cellular and cytokine content between vehicle- and APCP-treated group, apart from MCP-1 whose levels in APCP group were still higher than those in vehicle group.

It is known that MCP-1 represents a chemokine that, in the pleural space, is produced by mesothelial and inflammatory

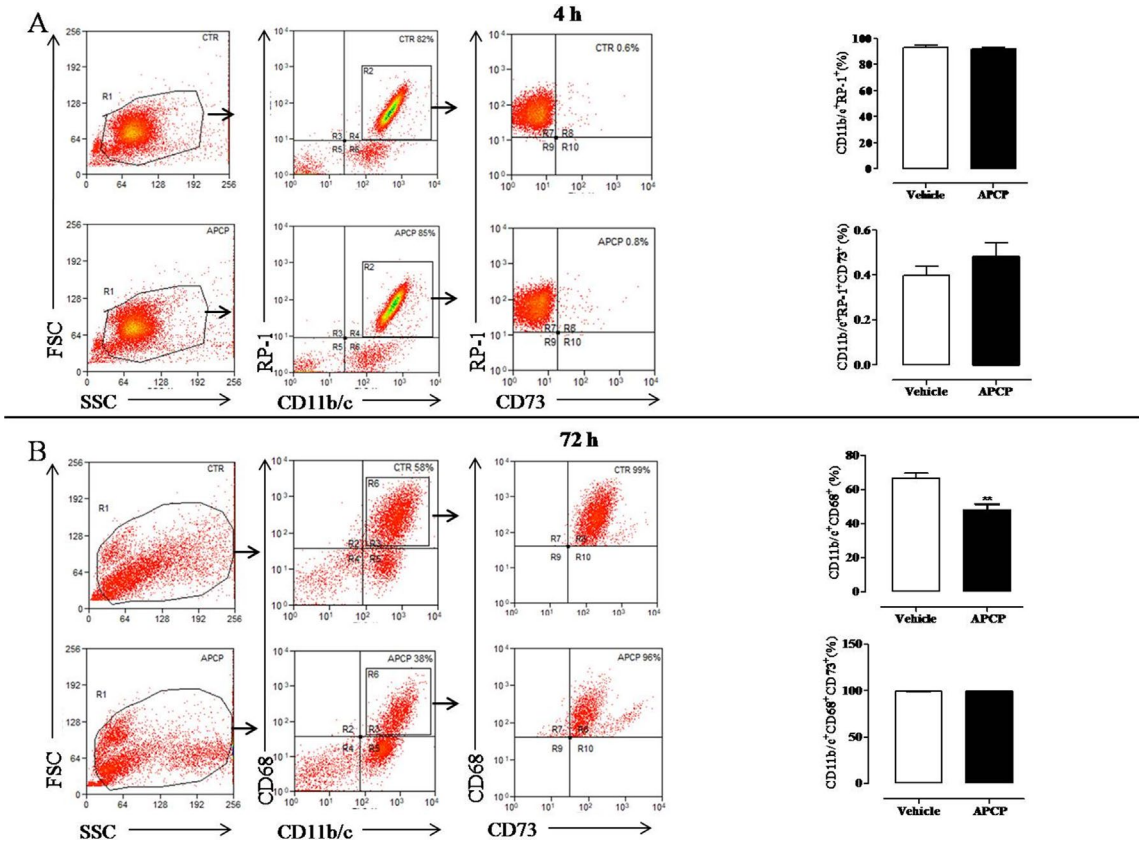

FIGURE 6 | Analysis of CD73 expression on leukocyte subpopulations recovered from the pleural cavity at 4 h **(A)** and 72 h **(B)** after carrageenan injection. Flow cytometry dot plots in panel A (left) show a representative experiment illustrating the gating strategy used to analyze CD11b/c positive (+) RP-1 positive (+) cells gated on leukocytes population identified by forward and side scatter characteristics. The CD73 expression was analyzed on CD11b/c$^+$ RP-1$^+$ cells. On the right of panel A, histograms summarize the percentage of cells positive to CD11b/c and RP-1 and the percentage of CD73 positive cells among CD11b/c$^+$ RP-1$^+$ cells in vehicle and APCP groups. Data are expressed as mean ± SE, N = 7. In panel B, flow cytometry dot plots are representative of CD11b/c$^+$ CD68$^+$ cells within leukocyte population identified by forward and side scatter characteristics. CD11b/c$^+$ CD68$^+$ cells were then analyzed for their CD73 expression. On the right of panel B, histograms summarize the percentage of cells positive to CD11b/c and CD68 and the percentage of CD73 positive cells among CD11b/c$^+$ CD68$^+$ cells in vehicle and APCP groups. Results represent mean ± SE, N = 7. **$p < 0.01$. Two-tailed Student's t-test.

cells (Deshmane et al., 2009; Batra and Antony, 2015). Recently, Lansley et al. (2017) have demonstrated that MCP-1 plays a crucial role in pleural effusion in a mouse model of carrageenan-induced pleurisy. Here, we show that MCP-1 is the cytokine that prevails in pleural lavage fluids at 4 and 72 h following carrageenan injection in rats; moreover, our results suggest that MCP-1 production is under the control of CD73. The role of CD73 in the control of neutrophil influx in response to inflammation and infections has also demonstrated in experiments performed in transgenic mice lacking CD73 enzyme, in which following *Mycobacterium tubercolosis* infection, an increased influx of PMNs but not of monocytes has been observed (Petit-Jentreau et al., 2015). Consistently, we found that treatment with APCP at the onset of inflammation affects the early phase (4 h), dominated by neutrophil influx, but not the sub-acute phase (72 h) of inflammation, dominated by monocyte influx. APCP is a well described CD73 inhibitor; however, there is evidence that this compound also inhibits human nucleotide pyrophosphatase/phosphodiesterases (NPPs), a related nucleotidase hydrolizing ATP or ADP to AMP although with very low potency (Bhattarai et al., 2015). NPP is also involved in the adenosine pathway recognized as non-canonical, leading to adenosine accumulation from ATP and NAD (Ferrero et al., 2019). Furthermore, there is evidence that APCP through increasing AMP levels may indirectly inhibit NTPDase (CD39) (Covarrubias et al., 2016). Nonetheless, what we have found is that treatment with APCP exacerbates carrageenan-induced pleurisy in rats; we claim that to this effect contributes the inhibition of CD73, and probably of the other enzymes involved in the adenosinergic pathway, with the resulting loss of the control that these ectoenzymes exert on the inflammatory environment. Thus, CD73, or more extensively the adenosinergic pathway, results to be critical for PMN trafficking in the model of carrageenan-induced pleurisy in rat. Similarly, it has been demonstrated that CD73 controls PMN trafficking in lung injury induced by bacterial lipopolysaccharide inhalation in mice (Reutershan et al., 2009).

We also attempted to analyze cells collected at 4 h, to evaluate how they behaved far from the inflammatory microenvironment.

For this purpose, we evaluated cell migration *ex vivo*, in presence or not of fMLP as chemoattractant agent. Interestingly, we found that PMNs collected from APCP-treated animals showed increased ability to migrate, even spontaneously, in absence of fMLP, compared to cells obtained from vehicle group. These results suggest that early inhibition of CD73 makes inflammatory cells, mostly neutrophils, prone to migrating. Likely, this effect may depend upon a contact with elevated levels of cytokines of cells obtained from APCP-treated animals compared to cells obtained from inflamed vehicle-treated animals. Alternatively, we might hypothesize that when CD73 is inhibited, a phenotypically different neutrophil population accumulates at the site of inflammation.

Neutrophil heterogeneity has been described (Scapini et al., 2016), and it is known that their differentiation toward distinct subpopulations depends upon environmental signals and also their lifespan (Silvestre-Roig et al., 2016). It is known that CD73 expression on neutrophils is normally low, and it's under the control of inflammatory cytokines (Szabo and Pacher, 2012; Antonioli et al., 2013). By performing flow cytometric analysis of cells accumulated into the pleural space, we found that only a very small percentage of all leukocytes accumulated 4 h following carrageenan injections were $CD73^+$, conversely, almost the totality of leukocytes accumulated at 72 h were $CD73^+$, and there was no difference in the percentage of cells $CD73^+$ between vehicle- and APCP-treated group. Nonetheless, there was difference in AMPase activity between cells obtained from the two groups, being the activity of cells recovered from pleural lavage fluid of APCP-treated rats significantly lower than AMPase activity of cells from vehicle-treated group. All together, these results suggest that cell trafficking into the pleural space is regulated by CD73 enzymatic activity. Our data agree with previous experimental results, in mice, and clinical data that highlight the important regulatory role of CD73 in the control of cell trafficking and cytokine production in an inflammatory environment (Reutershan et al., 2009; Petrovic-Djergovic et al., 2012; Al-Taei et al., 2016).

However, to better understand the mechanism by which CD73/adenosine pathway controls the inflammatory reaction in our model, we need to establish which cells are targeted by APCP. Indeed, we found that the effect of rat treatment with APCP was evident at 4 hours, when only a very small subset of infiltrating cells were $CD73^+$; thus, it is conceivable that APCP does not exert its effect on recruited cells but on stromal cells and/or on other type of cells, such as dendritic cells, representing a first line of host defense and demonstrating to infiltrate the pleural space (Condon et al., 2011). It is known that dendritic cells expressing both CD39 and CD73 transform a proinflammatory ATP-rich microenvironment into an antinflammatory adenosine rich environment (Silva-Vilches et al., 2018). Unfortunately, we did not sort dendritic cells by flow cytometry; nonetheless, it can be argued that subset of cells $CD11b/c^+CD68^-$ found at 72 hours are dendritic cells; however, this point needs to be further investigated.

In conclusion, rat treatment with APCP exacerbates the early phase of inflammation by controlling pleural effusion and polymorphonuclear migration *in vivo* and *ex vivo*. Although we cannot rule out the possibility that APCP inhibits other ectoenzymes involved in the adenosinergic pathway, we argue that CD73 inhibition might play a major role. On this basis, we suggest that CD73 might represent a valid biomarker for pleural effusion besides being a potential target for novel therapeutic interventions.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All procedures were performed according to the Italian and European regulations (DL 26/2014) on the protection of animals used for experimental and other scientific purposes and were approved by Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

EC, SM, and CC contributed to the study design, study conduct, and data analysis. EC and CC contributed to the data collection, data interpretation and drafting of the manuscript. EC, RC, AI, and CC revised the manuscript.

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The Ionotropic P2X4 Receptor has Unique Properties in the Heart by Mediating the Negative Chronotropic Effect of ATP While Increasing the Ventricular Inotropy

*Bruno Bragança[1,2], Sílvia Nogueira-Marques[1], Fátima Ferreirinha[1], Ana Patrícia Fontes-Sousa[1] and Paulo Correia-de-Sá[1]**

[1] Laboratório de Farmacologia e Neurobiologia, Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal, [2] Hospital Pedro Hispano, ULS Matosinhos, Matosinhos, Portugal

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***Correspondence:**
Paulo Correia-de-Sá
farmacol@icbas.up.pt

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Background: Mounting evidence indicate that reducing the sinoatrial node (SAN) activity may be a useful therapeutic strategy to control of heart failure. Purines, like ATP and its metabolite adenosine, consistently reduce the SAN spontaneous activity leading to negative cardiac chronotropy, with variable effects on the force of myocardial contraction (inotropy). Apart from adenosine A_1 receptors, the human SAN expresses high levels of ATP-sensitive ionotropic P2X4 receptors (P2X4R), yet their cardiac role is unexplored.

Methods: Here, we investigated the activity of P2 purinoceptors on isolated spontaneously beating atria (chronotropy) and on 2 Hz-paced right ventricular (RV, inotropy) strips from Wistar rats.

Results: ATP (pEC_{50} = 4.05) and its stable analogue ATPγS (pEC_{50} = 4.69) concentration-dependently reduced atrial chronotropy. Inhibition of ATP breakdown into adenosine by NTPDases with POM-1 failed to modify ATP-induced negative chronotropy. The effect of ATP on atrial rate was attenuated by a broad-spectrum P2 antagonist, PPADS, as well as by 5-BDBD, which selectively blocks the P2X4R subtype; however, no effect was observed upon blocking the A_1 receptor with DPCPX. The P2X4R positive allosteric modulator, ivermectin, increased the negative chronotropic response of ATP. Likewise, CTP, a P2X agonist that does not generate adenosine, replicated the P2X4R-mediated negative chronotropism of ATP. Inhibition of the Na^+/Ca^{2+} exchanger (NCX) with KB-R7943 and ORM-10103, but not blockage of the HCN channel with ZD7288, mimicked the effect of the P2X4R blocker, 5-BDBD. In paced RV strips, ATP caused a mild negative inotropic effect, which magnitude was 2 to 3-fold increased by 5-BDBD and KB-R7943. Immunofluorescence confocal microscopy studies confirm that cardiomyocytes of the rat SAN and RV co-express P2X4R and NCX1 proteins.

Conclusions: Data suggest that activation of ATP-sensitive P2X4R slows down heart rate by reducing the SAN activity while increasing the magnitude of ventricular contractions. The mechanism underlying the dual effect of ATP in the heart may involve inhibition

of intracellular Ca^{2+}-extrusion by bolstering NCX function in the reverse mode. Thus, targeting the P2X4R activation may create novel well-tolerated heart-rate lowering drugs with potential benefits in patients with deteriorated ventricular function.

Keywords: ATP, P2X4 receptor, Na^+/Ca^{2+} exchanger, sinoartrial node, spontaneously beating atria, paced right ventricle

INTRODUCTION

Heart rate is primarily set in the right atria by spontaneous generation of rhythmic actions potentials in the sinoatrial node (SAN) (Boyett et al., 2000). The autonomous activity of SAN cardiomyocytes is orchestrated by activation of several ion channels and regulating proteins, which interplay to generate effective action potentials in a regular time basis (Yaniv et al., 2015; Fabbri et al., 2017). The unstable resting membrane potential characteristic of SAN cardiomyocytes is mainly due to mutual influence of intracellular Ca^{2+} "clocks" and membrane potential oscillations. The unstable resting membrane potential and the spontaneous firing of SAN cardiomyocytes are mainly attributed to "funny" currents carried by hyperpolarization-activated cyclic nucleotide-gated channels (HCN) and by the electrogenic Na^+/Ca^{2+} exchanger (NCX) functioning in the forward Ca^{2+}-extrusion mode (Tsutsui et al., 2018; Vinogradova et al., 2018). NCX operates as an integrator of intracellular Ca^{2+} with the membrane potential, in a way that it is responsible by a slow depolarizing current that drives the diastolic depolarization phase in response to calcium leakage from sarcoplasmatic reticulum and other subsidiary Ca^{2+} stores (Bogdanov et al., 2001; Sanders et al., 2006; Groenke et al., 2013; Herrmann et al., 2013). The NCX dynamically fluctuates between forward and reverse modes, thereby extruding or importing Ca^{2+} to subcellular regions, respectively (Samanta et al., 2018). Impairment of the NCX forward-mode by genetic ablation, pharmacological inhibition or even by simply changing its electrochemical gradient consistently induces bradycardia, supporting NCX as a key element in SAN pacemaker activity (Kurogouchi et al., 2000; Bogdanov et al., 2001; Sanders et al., 2006; Groenke et al., 2013; Herrmann et al., 2013).

The SAN, like other regions of the heart, is under control of countless number of signaling molecules, including adenosine triphosphate (ATP) and its derivatives, namely adenosine (Erlinge and Burnstock, 2008; Mangoni and Nargeot, 2008; Burnstock and Pelleg, 2015). Since the pioneering work of Drury and Szent-Gyorgyi almost a century ago (1929), the protective role of purine nucleotides and nucleosides as retaliatory mediators released in response to hypoxia and stressful stimuli in the vascular system has been expanded to the myocardium (Forrester and Williams, 1977), where purines engage energy-saving negative chronotropic, dromotropic and inotropic actions (Versprille and van Duyn, 1966; Lundberg et al., 1984; Pelleg et al., 1987; Belardinelli et al., 1995). Apart from cellular damage, ATP release is not stochastic but rather a fine regulated process (Lazarowski, 2012), involving (1) electrodiffusional movement through membrane ion channels, including pannexin- and connexin-containing hemichannels; (2) facilitated diffusion by nucleotide-specific ATP-binding cassette (ABC) transporters; and (3) cargo-vesicle trafficking and exocytotic granule secretion (Clarke et al., 2009; Pinheiro et al., 2013a; Pinheiro et al., 2013b; Timoteo et al., 2014).

Once in the extracellular milieu, ATP builds up its effects through activation of P2 purinoceptors, which comprise seven ionotropic (P2X1-7) and eight metabotropic ($P2Y_{1,2,4,6,11,12,13,14}$) receptors. Extracellular ATP can also indirectly activate purinoceptors of the P1 family (A_1, A_{2A}, A_{2B}, and A_3) after its conversion into adenosine by cascade of ectonucleoside triphosphate diphosphohydrolases (NTPDases) (Yegutkin, 2008). Intravenous ATP produces negative chronotropy, as well as several other acute cardiovascular effects that resembles adenosine application, almost neglecting ATP-sensitive receptors as active and functional regulators of the cardiac function (Pelleg and Belhassen, 2010). Apart from a massive presence of adenosine A_1 receptors (Braganca et al., 2016), the SAN from both rodents and humans expresses several P2 purinoceptor subtypes, including ionotropic P2X4, P2X7, and metabotropic $P2Y_1$, $P2Y_2$, and $P2Y_{14}$ (Musa et al., 2009), yet their role is still largely unknown. Besides species differences, the type and relative abundance of P2 purinoceptors in the heart varies with location and disease conditions (Musa et al., 2009).

Relatively recent evidence called our attention showing that the highly expressed ionotropic P2X4 in the human SAN may be an important modulator of NCX function (Shen et al., 2014). Activation of ionotropic P2X receptors, including the P2X4, triggers the influx of Na^+ that is subsequently exchanged by Ca^{2+} *via* the NCX (Jarvis and Khakh, 2009). Using various models of heart failure in rodents, it has been demonstrated that reversion of the NCX function by P2X4 activation may lead to improvements of ventricular performance and clinical outcome (Hu et al., 2001; Mei and Liang, 2001; Yang et al., 2004; Yang et al., 2014; Yang et al., 2015). Indeed, pharmacological inhibition of Na^+/K^+-ATPase with digitalis interferes with cardiac NCX function, resulting in both negative chronotropy and positive inotropy, thus explaining the rationale for usage of digitalis-like drugs in the management of patients with tachyarrhythmias and ventricular contractile incompetence (Blomstrom-Lundqvist et al., 2003; Ponikowski et al., 2016).

In this study, we aimed at characterizing the effect of the P2X4 on heart rate using isolated spontaneously beating rat atria strips. Taking into consideration that most currently available negative chronotropic drugs are associated with a negative inotropic impact as a major drawback (Ponikowski et al., 2016), experiments were designed to assess the effect of the P2X4 on the magnitude of paced ventricular contractions.

MATERIALS AND METHODS

Animals

Animals care and experimental procedures were conducted in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), Directive 2010/63/EU and Portuguese rules (DL 113/2013). All experimental protocols involving animals were approved by the competent national authority Direção Geral de Alimentação e Veterinária, and by the ICBAS Animal Ethical Committee (No. 224/2017). All efforts were made to minimize animal suffering and to reduce the number of animals used according to the ARRIVE guidelines. Wistar rats (*Rattus norvegicus*; 250–300 g; Charles River, Barcelona, Spain) of either sex were kept at a constant temperature (21 °C) and a regular light (06:30–19:30 h) – dark (19:30–06:30 h) cycle, with food and water provided *ad libitium*.

Isolated Spontaneously Beating Atria

Isolated spontaneously beating atria were prepared using a previously described method (Braganca et al., 2016), with some modifications. In brief, hearts were rapidly excised after decapitation followed by exsanguination (Rodent guillotine, Stoelting 51330), and placed in a physiological solution (Tyrode's solution) composed of (mM): NaCl 137; KCl 4.7; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 0.4; $NaHCO_3$ 11.9; glucose 11.2 and gassed with 95% O_2 + 5% CO_2 (at pH 7.4). Hearts were allowed to beat freely for a few seconds at room temperature, to empty its blood content. The paired rat atria with the SAN region were dissected out, cleaned of fatty tissues, and suspended in a 14-ml organ bath containing gassed Tyrode's solution at 37 °C. Each auricular appendage was tied and connected with thread to the organ bath wall and to an isometric force transducer (MLT050/D; AD Instruments, Colorado Springs, CO, USA). Changes in isometric tension were recorded continuously using a PowerLab data acquisition system (Chart 5, version 4.2; AD Instruments, Colorado Springs, CO, USA). The preparations were allowed to equilibrate for 30–40 min. During this time, the preparations were continuously superfused with Tyrode's solution (1 ml/min) and the tension was adjusted to 9.8 mN. This procedure allows atria (with intact SA node) to progressively recover rhythmic spontaneous beatings (average of 247 ± 5 beats min^{-1} at the beginning of the experimental protocol, n = 79); preparations with spontaneous atrial rate below 200 beats min^{-1} or exhibiting rhythm variations above 10 beats min^{-1} during equilibrium were discarded to ensure measurements were made in atria with intact primary pacemaker SAN activity. None of the preparations exhibited noticeable signs of ectopic-activity caused by secondary pacemakers, usually related to asynchronous and abnormal contractions.

Under these experimental conditions, spontaneously beating rat atria respond to muscarinic and β-adrenergic stimulation, but are unaffected by the application of atropine or atenolol alone used in concentrations high enough (1 μM and 3 μM, respectively) to prevent the effects of acetylcholine (100 μM) and isoproterenol (30 nM), respectively (data not shown). Thus, myographic recordings reported in this study include rate (chronotropic effect) and contractile force (inotropic effect) of spontaneously beating atria measured in the absence of cholinergic and/or adrenergic tone.

Isolated Paced Right Ventricle Strips

Following the isolation procedures described above for spontaneously beating atria, we also obtained right ventricular (RV) strips (2 mm wide, 8–10 mm long and 1.5 mm thick) by cutting RV-free wall longitudinally to its surface. A pair of ventricular strips was used from each right ventricle. RV strip ends were tied and connected with thread to the 14-ml organ bath hook and to an isometric force transducer (MLT050/D; AD Instruments, Colorado Springs, CO, USA). Changes in the isometric tension of RV strips, measured both by the active tension (mN/mg of wet tissue weight) and by the derivative of developed force over time (+dF/dt, mN/s), were tested at a fixed frequency of 120 beats per min commanded by electric field stimulation of the preparations, so that inotropy was measured without being affected by concurrent changes in chronotropy. Electric pacing (2 Hz, +50% voltage above threshold, 2 ms) was generated by independent Grass S48 stimulators (Quincy, MA, USA) and delivered *via* two platinum electrodes positioned on each side of the preparations. Equilibrium of the preparations was performed as described above for spontaneously beating atria. Only ventricular preparations exhibiting rhythmic contractions with similar amplitude were used.

Experimental Design

After reaching a steady-state, the Tyrode's solution flow through the organ bath was stopped and the preparations were incubated for an additional period of 15 min before drug applications. The concentration–response curves for ATP and related nucleotides were performed by non-cumulative application of increasing concentrations of the nucleotides during 5 min followed by a washout period with Tyrode's solution (15 ml/min) to avoid biases resulting from accumulation of their metabolites and to prevent receptors desensitization. To shorten the experimental duration and, thus, to increase results reproducibility, the majority of the preparations were incubated for 5 min with a fixed concentration (near the EC_{50} value) of the nucleotide either in the absence and in the presence of drug modulator (e.g. receptor antagonist, channel inhibitor); the latter contacted with the preparations at least for 15 min before application of the nucleotide and we kept 2-h washout intervals between testing again the same nucleotide to exclude biases related to P2 purinoceptors desensitization (controls not shown). To avoid damage of RV strips performance by prolonged pacing at 2 Hz, parallel experiments were performed using the two strips coming from the same animal to test the effect of ATP in the absence and in the presence of any modulator.

Immunofluorescence Staining and Confocal Microscopy Studies

Rat hearts were excised (see above) and placed in oxygenated Tyrode's solution at 33–34°C. Following heart excision, the right atrium (RA) containing the SAN region and RV were accurately

isolated through the interauricular and interventricular septa and cleaned from tissue debris. Tissue fragments were placed over a small lung lobule fragment with the endocardial layer facing down, stretched to all directions, pinned flat onto cork slices and embedded in Shandon cryomatrix (Thermo Scientific) before frozen in a liquid nitrogen–isopentane; frozen samples were stored at −80ºC until use. Frozen sections with 8 µm thickness were cut perpendicular to the crista terminalis of the RA and parallel to the long axis in the case of RV (see Braganca et al., 2016). Following fixation, the preparations were washed three times for 10 min each using 0.1 M PBS and incubated with a blocking buffer, consisting in fetal bovine serum 10%, bovine serum albumin 1%, Triton X-100 0.3% in PBS, for 2 h. After blocking and permeabilization, samples were incubated with selected primary antibodies (**Table 1**) diluted in incubation buffer (fetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), overnight at 4ºC. For double immunostaining, antibodies were combined before application to tissue samples. Following the washout of primary antibodies with PBS (3 cycles of 10 min) tissue samples were incubated with species-specific secondary antibodies (**Table 1**) in the dark for 2 h, at room temperature. Finally, VectaShield mounting medium with 4′-6-diamidino-2-phenylindole (DAPI) to stain the nuclei (H-1200; Vector Labs) was used, before cover-slipping the glass slides. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus Fluo View, FV1000, Tokyo, Japan).

The SAN was characterized as described in a previous study from our group (Braganca et al., 2016). SAN identification was also facilitated by observation of the sinus node artery surrounded by small-size cardiomyocytes positive for the hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) and negative against connexin-43 (Cx43), a gap junction protein ubiquitously expressed in the heart apart from in nodal tissue. In some of the experiments, the existence of a large number of neurofilament 160 (NF-160) positive neuronal fibers was also used to identify the SAN region (Tellez et al., 2006; Braganca et al., 2016).

Solutions and Chemicals

Adenosine-5′-triphosphate (ATP); adenosine 5′-[γ-thio]-triphosphate(ATPγS);cytidine-5′-triphosphate(CTP);1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX); 22,23-dihydroavermectin B1 (ivermectin); and 2-[(3,4-dihydro-2-phenyl-2H-1-benzopyran-6-yl)oxy]-5-nitro-pyridine (ORM-10103) were obtained from Sigma (Poole, U.K.). 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD); 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079); 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KR-R7943) and sodium metatungstate (POM-1) were from Tocris Cookson Inc. (Bristol, UK). Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and ZD7288 were from Ascent Scientific (Bristol, UK). Dimethylsulfoxide (DMSO), serum albumin and Triton X-100 were from Merck (Darmstadt, Germany). DPCPX was made up in 99% DMSO/1% NaOH 1 mM (v/v); 5-BDBD, A438079, Ivermectin, ORM-10103, KB-R7943 were made up in DMSO. Other drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily and kept protected from the light to prevent photodecomposition. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v), were observed. The pH of the Tyrode's solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

Presentation of Data and Statistical Analysis

The isometric contractions were recorded and analyzed before and after the addition of each drug at the desired concentration. Results were presented as percentages of variation compared to baseline (% Δ baseline), obtained before application of the test drug. Data are expressed as mean ± SEM, with *n* indicating the number of animals used for a particular group of experiments. Graphical data are expressed as box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage of variation from baseline. Since modulation of ATP-mediated responses were unpredictable, we did not apply any power calculations to pre-determine sample size, thus we pre-specified a number of 4–7 experiments for each condition. Concentration–response curves were analyzed by fitting four-parameter logistic sigmoidal functions to the experimental data to estimate pEC_{50} for negative chronotropy and inotropy of the nucleotides. All curve fitting procedures, graphical, and

TABLE 1 | List of primary and secondary antibodies used in immunohistochemistry experiments.

Antigen	Code	Host	Dilution	Supplier
Primary antibodies				
Cx43	Ab11370	Rabbit (rb)	1:700	Abcam
NF-160	Ab7794	Mouse (ms)	1:1,000	Abcam
HCN4	Agp-004	Guinea-pig (gp)	1:150	Alomone
P2X4 (C-terminus)	Apr-002	Rabbit (rb)	1:200	Alomone
P2X4 (extracell. loop)	Apr-024	Rabbit (rb)	1:200	Alomone
NCX1	Anx-011	Rabbit (rb)	1:50	Alomone
Secondary antibodies				
Alexa Fluor 488 anti-rb	A-21206	Donkey	1:1,500	Molecular probes
Alexa Fluor 568 anti-ms	A-10037	Donkey	1:1,500	Molecular probes
TRITC 568 anti-gp	706-025-148	Donkey	1:150	Jackson Immuno Res.

statistical analyses were carried out using GraphPad Prism 7.04 for Windows software (La Jolla, USA). Spontaneous or electrically-evoked mechanical tension (inotropic effect) and contraction rate (chronotropic effect) were evaluated using the Student's *t*-test for paired samples assuming a Gaussian distribution of data. Given that significant variability (ranging from 15 to 35%) was observed amongst animals for the negative chronotropic action of ATP (100 μM), changes on the nucleotide effect in the presence of a modulator was always compared to its absence in the same animal or preparation to make differences between paired values consistent. A value of $p < 0.05$ was considered to represent a significant difference.

RESULTS

Effects of ATP on Sinoatrial Chronotropy and Right Ventricular Inotropy

Non-cumulative application of ATP (0.001–1 mM) concentration-dependently decreased atrial chronotropy and right ventricular inotropy (**Figure 1**). The onset of ATP response was readily visible in about 30 s for both myocardial preparations; it reached a sustained maximal effect roughly 1 min after application and lasted while the nucleotide was kept in the incubation fluid, *i.e.* at least for 5 min. Spontaneously beating atria were slightly more sensitive ($p < 0.05$) to the inhibitory effect of ATP compared to paced RV strips (**Figure 1C**). The estimated pEC_{50} for the negative chronotropic and inotropic ATP responses were 4.05 and 3.45, respectively. Of note, ATP exhibited a biphasic effect on atrial inotropy, which was characterized by an initial decrease in the magnitude of atrial contractions followed by a gradual recovery to levels above the baseline (**Figure 1A**), as reported by other authors (Froldi et al., 1994; Gergs et al., 2008).

The Negative Chronotropic Effect of ATP Depends of P2 Purinoceptors Activation

The negative chronotropic effect of ATP following intravenous application of the nucleotide resembles that obtained after administration of adenosine (Pelleg and Belhassen, 2010), the end product of ATP hydrolysis by the ectonucleotidase cascade (Cardoso et al., 2015). To know whether ATP is acting directly on P2 purinoceptors or indirectly *via* P1 receptors after its extracellular conversion into adenosine, we tested the effect of ATPγS, an enzymatically-stable ATP analogue. ATPγS (100 μM) decreased sinoatrial chronotropy (−19 ± 5%, n = 6) by a similar extent to that observed for ATP (100 μM, −18 ± 5%, n = 6) (**Figure 2A**); the estimated pEC_{50} for the negative chronotropic effect of ATPγS was 4.69 (**Supplementary Figure S1A**). Moreover, the broad-spectrum P2 receptor antagonist PPADS (10 μM) significantly attenuated the negative chronotropic response of ATP (−20 ± 2% vs −11 ± 4%, n = 7, $p < 0.001$) (**Figure 2B**); the blocking effect of PPADS was more evident upon increasing the concentration of the P2 receptor antagonist to 100 μM (−17 ± 4% vs −2 ± 2%, n = 4) (see **Supplementary Figure S1D**).

Blockage of ATP breakdown by NTPDases with POM-1 (100 μM) significantly ($p < 0.05$) potentiated the negative chronotropic response of ATP (100 μM) (−19 ± 6% vs −35 ± 8%, n = 5, $p < 0.05$) (**Figure 2C**), whereas the selective adenosine A_1 receptor antagonist, DPCPX (3 nM) (Lohse et al., 1987), was ineffective (−19 ± 3% vs −21 ± 4%, n = 5; $p > 0.05$) (**Figure 2D**). Indeed, the NTPDase inhibitor, POM-1 (100 μM), shifted to the left ($pEC_{50} = 5.10$; $p < 0.05$) the concentration-response curve of ATP (0.001–1 mM) without significantly modifying the Hill slope (see **Supplementary Figure S1B**).

On their own, PPADS (10 μM and 100 μM), POM-1 (100 μM) and DPCPX (3 nM) were virtually devoid of effect on spontaneous atrial beating rate (see **Supplementary Figure S2**). It is also worth noting that blockage of muscarinic acetylcholine

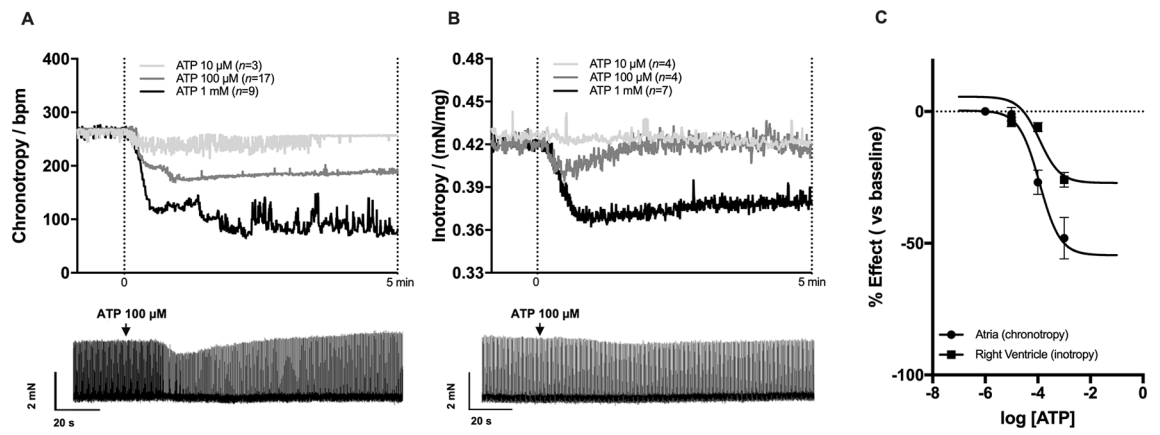

FIGURE 1 | Effects of ATP on spontaneously beating atria (**A**, chronotropy, beats min^{-1}) and 2 Hz-paced right ventricular (**B**, inotropy, mN/mg of tissue) strips from Wistar rats. ATP (0.01–1 mM) was applied non-cumulatively for 5 min followed by a washout period to avoid biases resulting from bath accumulation of metabolites and from receptors desensitization. Upper panels **(A)** and **(B)** show average values; bottom panels show typical myographical recordings of atrial and ventricular preparations challenged with ATP (100 μM). Panel **(C)** shows the concentration-response curves for ATP (0.001–1 mM) fitted by four-parameter logistic sigmoidal functions used to estimate pEC_{50} values for the negative chronotropic and inotropic effects of the nucleotide. Data are expressed as mean ± SEM from an *n* number of animals indicated in upper panels **(A)** and **(B)**, respectively.

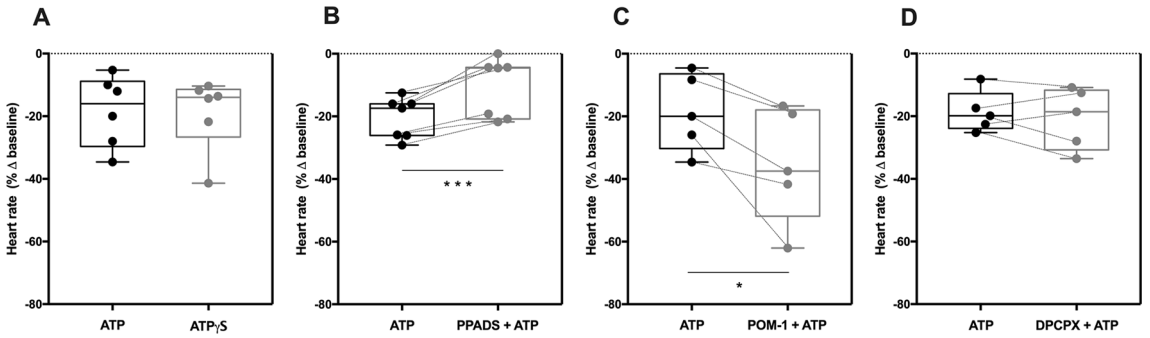

FIGURE 2 | The negative chronotropic effect of ATP depends of P2 purinoceptors activation. The negative chronotropic effect of ATP (100 μM) was tested either in the absence or in the presence of the non-selective P2 receptor antagonist, PPADS (10 μM, **B**), the NTPDase inhibitor, POM-1 (100 μM, **C**), and the selective adenosine A_1 receptor antagonist, DPCPX (3 nM, **D**). The negative chronotropic effect of the enzymatically stable ATP analogue, ATPγS (100 μM, **A**), is also shown for comparison. Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. Each data point represents the result of a single experiment; data from the same experiment are connected by lines. *p < 0.05, ***p < 0.001 (Student's *t*-test for paired samples) represent significant differences when compared to the effect of ATP alone.

receptors with atropine (1 μM) did not modify the negative chronotropic effect of ATP (100 μM) (data not shown), ruling out putative changes in the cholinergic tone operated by ATP.

The Negative Chronotropic Effect of ATP Is Mediated by P2X4 Receptors Activation

The P2 purinoceptors expression in the SAN is species specific. For instance, in humans the rank order of expression of ionotropic P2X receptors is the following: P2X4 > P2X7> > P2X1 > P2X5 (P2X2 and P2X3 are absent), while in rats it is P2X5> > P2X7> > P2X4~P2X1~P2X2 > P2X3 (Musa et al., 2009). Regrettably, there are no specific pharmacologic agonists or antagonists to the P2X5 receptor. It is worth noting that the P2X7 receptor is negatively modulated by extracellular Ca^{2+} and shows low affinity (0.1–1mM) for ATP. This contrasts with the estimated EC_{50} values in the low micromolar range for ATP and ATPγS that is characteristic of the most abundant P2X4 receptor in the human SAN (Soto et al., 1996; Michel et al., 1997). Interestingly, both P2X4 and P2X7 receptor pores are able to translocate extensive amounts of Na^+ into the cells. These premises prompted us to test whether these receptors could be involved in the negative chronotropic effect of ATP.

Selective blockage of the P2X7 receptor with A438079 (3 μM, **Figure 3A**) failed to modify the negative chronotropy effect of ATP (100 μM) (−31 ± 5% vs −26 ± 9%, n = 6, $p > 0.05$), whereas the potent and selective P2X4 receptor antagonist, 5-BDBD (10 μM) (Coddou et al., 2019), significantly attenuated ATP-induced negative chronotropism (−31 ± 7% vs −17 ± 5%, n = 6, $p < 0.05$) (**Figure 3B**). The negative chronotropic action of ATP (100 μM) was potentiated by ivermectin (30 μM) (−16 ± 3% vs −25 ± 3%, n = 5, $p < 0.05$) (**Figure 3C**), a drug that acts as positive allosteric

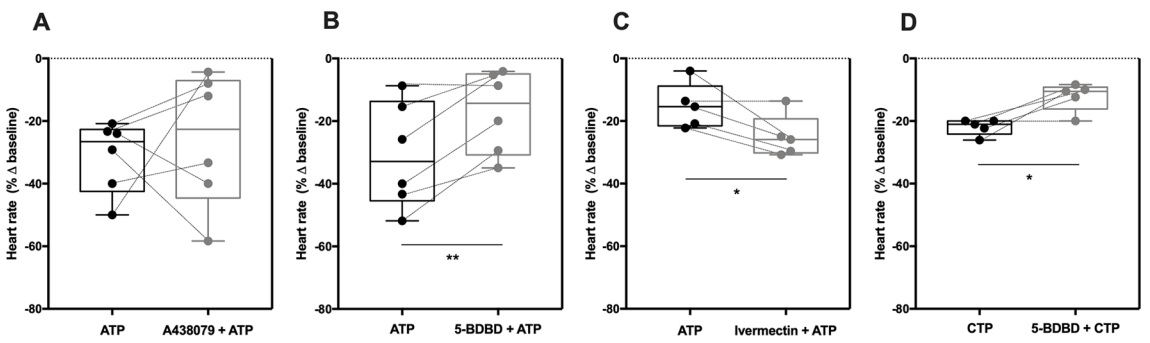

FIGURE 3 | The negative chronotropic effect of ATP is mediated by activation of P2X4. The negative chronotropic effect of ATP (100 μM) was tested either in the absence or in the presence of the P2X7 receptor antagonist, A438079 (3 μM, **A**), the P2X4 receptor antagonist, 5-BDBD (10 μM, **B**) and the positive allosteric modulator of the P2X4 receptor, ivermectin (30 μM, **C**). The negative chronotropic effect of CTP (1 mM, **D**) either in the absence or in the presence of 5-BDBD (10 μM), is also shown for comparison. Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. Each data point represents the result of a single experiment; data from the same experiment are connected by lines. *p < 0.05, **p < 0.01 (Student's *t*-test for paired samples) represent significant differences when compared to the effects of ATP or CTP alone, respectively.

modulator of the P2X4 receptor *via* a dual mechanism that involves potentiation and delayed inactivation of its currents, exhibiting selectivity over other P2X receptors (Khakh et al., 1999). The concentration-response curve of ATP (0.001–1 mM) was shifted to the left (pEC_{50} = 4.99; $p < 0.05$) by ivermectin (30 μM) compared to the effect of ATP alone (see **Supplementary Figure S1C**). Please note that, on their own, A438079 (3 μM, −5 ± 3%, n = 6), 5-BDBD (10 μM, −8 ± 9%, n = 5) and ivermectin (30 μM, 3 ± 3%, n = 5) were virtually devoid of effect on the spontaneous atrial frequency (see **Supplementary Figure S2**).

In order to further explore the potential involvement of the P2X4 receptor, we used CTP as a preferential P2X receptor agonist whose hydrolysis does not directly generate adenosine or other adenine nucleotides. Despite the fact that the P2X4 receptor exhibits low affinity for CTP compared to ATP (Soto et al., 1996; Kasuya et al., 2017), CTP (1 mM) decreased the spontaneous atrial rate by 22 ± 1% (n = 5) and this effect was also antagonized by 5-BDBD (10 μM, −12 ± 2%, n = 5, $p < 0.05$) (**Figure 3D**).

The P2X4-Mediated Negative Chronotropic Effect of ATP Involves NCX, But Not HCN

ATP binding to the P2X4 receptor dramatically increases Na^+ and Ca^{2+} influx through the receptor pore, which may interfere with NCX function as one of the sarcolemma controllers of the SAN pacemaker activity (Shen et al., 2014). Besides NCX, the normal sinus rhythm also depends on HCN channels mediating I_f currents (Bogdanov et al., 2001; Sanders et al., 2006; Groenke et al., 2013; Herrmann et al., 2013). Therefore, we thought it was relevant to evaluate the P2X4 receptor influence on downstream activation of NCX and/or HCN membrane transporters, which are essential to control heart rate. To this end, we used two different compounds known to inhibit NCX activity, namely KB-R7943 and the recently developed ORM-10103 (Jost et al., 2013). Since both inhibitors have putative negative chronotropic actions, we performed concentration–response curves to determine the minimal concentration beyond that reduction of chronotropy would be a problem in interaction experiments (data not shown). Incubations of KB-R7943 and ORM-10103 for 15 min at a final concentration of 3 μM had no effect on spontaneously beating atria strips (−3 ± 3%, n = 6, $p > 0.05$ vs baseline; −1 ± 2%, n = 5, $p > 0.05$ vs baseline; respectively) (**Supplementary Figure S2**).

The negative chronotropic effect of ATP (100 μM) was attenuated by KB-R7943 (3 μM; −19 ± 4% vs −8 ± 3%, n = 6, $p < 0.05$) and by ORM-10103 (3 μM; −29 ± 4% vs −17 ± 5%, n = 5, $p < 0.05$) (**Figures 4A**, **B**); the inhibitory effect of these compounds had a similar magnitude to that observed with the P2X4 antagonist, 5-BDBD (10 μM) (see **Figure 3B**). Pre-incubation with the ivabradine-like HCN channel inhibitor ZD7288 (300 nM) did not significantly modify the chronotropic effect of ATP (100 μM) (−12 ± 2% vs −11 ± 3%, n = 5, $p > 0.05$) (**Figure 4C**). On its own, ZD7288 (300 nM) decreased the spontaneous atrial rate only by 6 ± 2% below the control (n = 5, $p < 0.05$) (**Supplementary Figure S2**).

P2X4-Induced NCX Transport Reversal Counteracts the Negative Inotropic Effect of ATP in Paced Ventricular Strips

Apart from digitalis, heart rate slowing drugs used in clinical practice decrease cardiac inotropism as a major drawback (Ponikowski et al., 2016). As shown in **Figure 1B**, ATP (0.001–1 mM) concentration-dependently decreased the amplitude of paced RV contractions. **Figure 5** shows that blockage of P2X4 receptors with 5-BDBD (10 μM) augmented the negative inotropic effect of ATP (100 μM) measuring the percent variation of the active tension (−6 ± 2% vs −19 ± 5%, n = 5, $p < 0.05$; **Figure 5A**) or of the derivative of developed force over time (+dF/dt) (−4 ± 2% vs −18 ± 6%, n = 5, $p < 0.05$; **Figure 5B**) in paced RV strips. The effect of 5-BDBD (10 μM) was mimicked by KB-R7943 (3 μM), *i.e.* inhibition of NCX

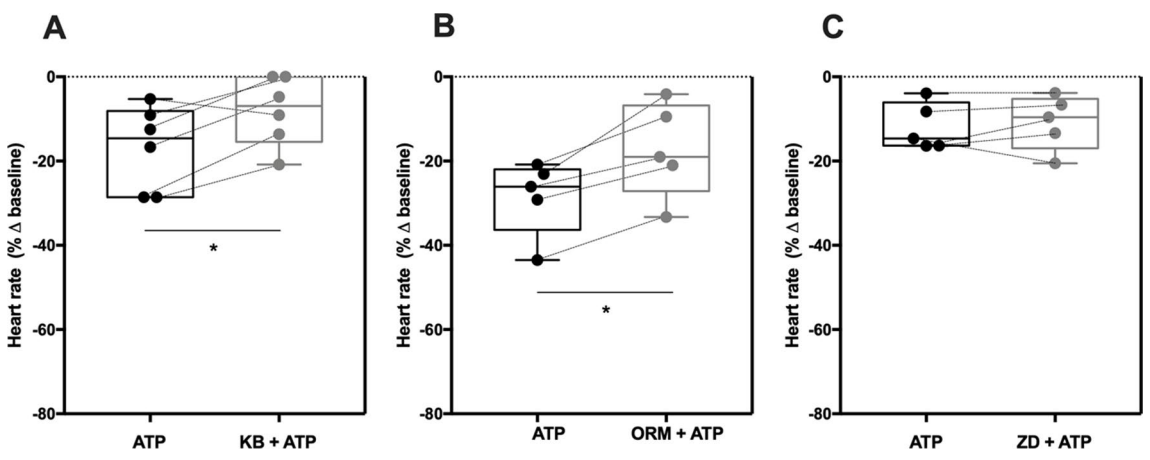

FIGURE 4 | The negative chronotropic effect of ATP involves ion exchange *via* pacemaker NCX, but not HCN. ATP (100 μM)-induced negative chronotropism was tested either in the absence or in the presence of two NCX inhibitors, KB-R7943 (3 μM, **A**) and ORM-10103 (3 μM, **B**), and of a HCN channel inhibitor, ZD7288 (300 nM, **C**). Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. Each data point represents the result of a single experiment; data from the same experiment are connected by lines. *p < 0.05 (Student's *t*-test for paired samples) represent significant differences when compared to the effect of ATP alone.

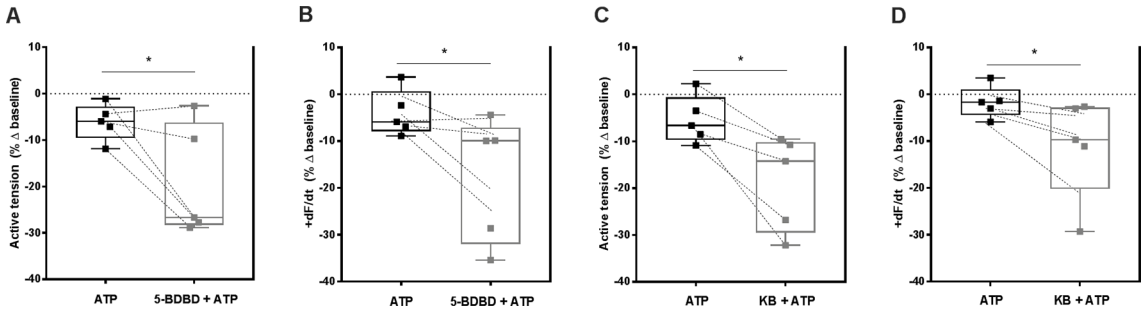

FIGURE 5 | Selective blockage of P2X4 and of NCX transporter partially offsets the negative inotropic effect of ATP in paced rat ventricular strips. The negative inotropic effect of ATP (100 μM) was tested either in the absence or in the presence of the P2X4 receptor antagonist, 5-BDBD (10 μM, **A** and **B**) and of the NCX inhibitor, KB-R7943 (3 μM, **C** and **D**). Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from the baseline isometric tension of RV strips, measured as the active tension (mN/mg of wet tissue weight, panels **A** and **C**) and the derivative of developed force over time (+dF/dt, mN/s, panels **B** and **D**); horizontal lines inside boxes indicate the corresponding medians. Each data point represents the result of a single experiment; data from the same experiment are connected by lines. *$p < 0.05$ (Student's *t*-test for paired samples) represent significant differences when compared to the effect of ATP alone.

sensitized RV strips to the negative inotropic effect of ATP (100 μM) calculated also measuring the percent variation of the active tension (−5 ± 2% vs −19 ± 5%, n = 5, $p < 0.05$; **Figure 5C**) or of the derivative of developed force over time (+dF/dt) (−2 ± 2% vs −12 ± 4%, n = 5, $p < 0.05$; **Figure 5D**). On their own, 5-BDBD (10 μM) and KB-R7943 (3 μM) marginally reduced ventricular inotropy by 14 ± 4% (n = 5, $p < 0.05$) and by 11 ± 19% (n = 5, $p > 0.05$), respectively (**Supplementary Figure S3**). These findings suggest that activation of the P2X4 partially counteracts the negative inotropic effect of ATP probably by reversing the NCX electrogenic current to pump Na^+ out and Ca^{2+} into ventricular cardiomyocytes.

Localization of P2X4, NCX1, and HCN4 Proteins in the Rat Heart

Confocal micrographs shown in **Figure 6** demonstrate that P2X4 receptor protein is expressed in the plasma membrane of cardiomyocytes of all assayed regions of the rat heart; in these experiments we used a knock-out validated antibody targeting the amino acid residues 370–388 of the C-terminus of the rat P2X4 receptor (Apr-002 from Alomone). Using tissues prepared in identical conditions and visualized with the same acquisition settings, one may conclude that the P2X4 receptor expression is higher in the SAN followed by the RV and RA. This regional

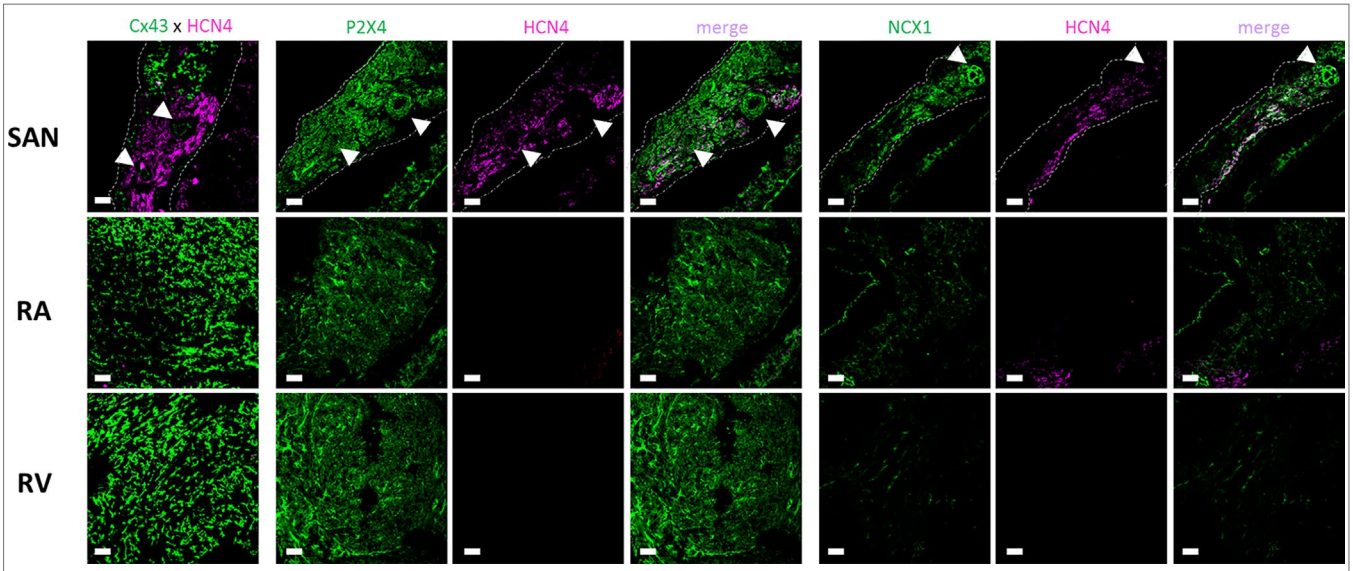

FIGURE 6 | Representative confocal micrographs showing the immunolocalization of the P2X4 receptor (Apr-002, C-terminus, Alomone) and NCX1 (Anx-011, Alomone) protein in the sinoatrial node (SAN), right atria (RA) and right ventricle (RV). The SAN was identified based on its low Cx43 (green) and high HCN4 (magenta) protein expression (left hand-side images). Images were taken from whole-mount heart preparations including the three analyzed regions, SAN, RA and RV. Dashed lines represent boundaries of the SAN. The pulmonary parenchyma was used as a structural support to facilitate immunostaining of myocardial sections and it is visible in the bottom right quadrant of each SAN image. White arrows indicate blood vessels including the SAN artery. Scale bar 30 μm. Images are representative of three different individuals.

difference was confirmed using a distinct antibody targeting amino acid residues 301–313 of the extracellular loop of the rat P2X4 receptor (Apr-024 from Alomone) (**Supplementary Figure S4**).

The immunoreactivity against NCX1 protein followed the same staining pattern to that found for the P2X4 receptor; the strongest immunofluorescence signal was also found in the SAN followed by other regions of the rat heart (**Figure 6**). Cardiomyocytes of the SAN region staining positively against NCX1 also exhibit immunoreactivity against the HCN4 protein. The same occurred regarding co-localization of P2X4 and HCN4. Taking this into consideration, even though double immunolabelling against P2X4 and NCX1 was not possible because available antibodies were raised in the same species (rabbit), it looks like that the staining pattern obtained with both P2X4 and NCX1 antibodies indicates that they may co-localize in HCN4 positive cardiomyocytes of the SAN (**Figure 6**). Please note that the smooth muscular layer of SAN blood vessels also exhibits strong immunoreactivity against P2X4 and NCX proteins (**Figure 6**, arrow heads). Likewise, these two proteins also co-localize with neurofilament 160 (NF160) in neuronal fibers of the SAN region (**Supplementary Figure S5**).

DISCUSSION

Data suggest that activation of ATP-sensitive P2X4 receptors plays a major contribution in decreasing the spontaneous activity of the SAN while partially offsetting the negative inotropic effect of the nucleotide by downstream reversing the electrogenic NCX mode of function (**Figure 7**).

ATP-Sensitive P2X4 Receptors Decrease Sinoatrial Pacemaker Activity

Extracellular ATP is an endogenous regulator of the cardiovascular function by acting either directly on P2 receptors or indirectly on P1 receptors after its breakdown to adenosine by NTPDases

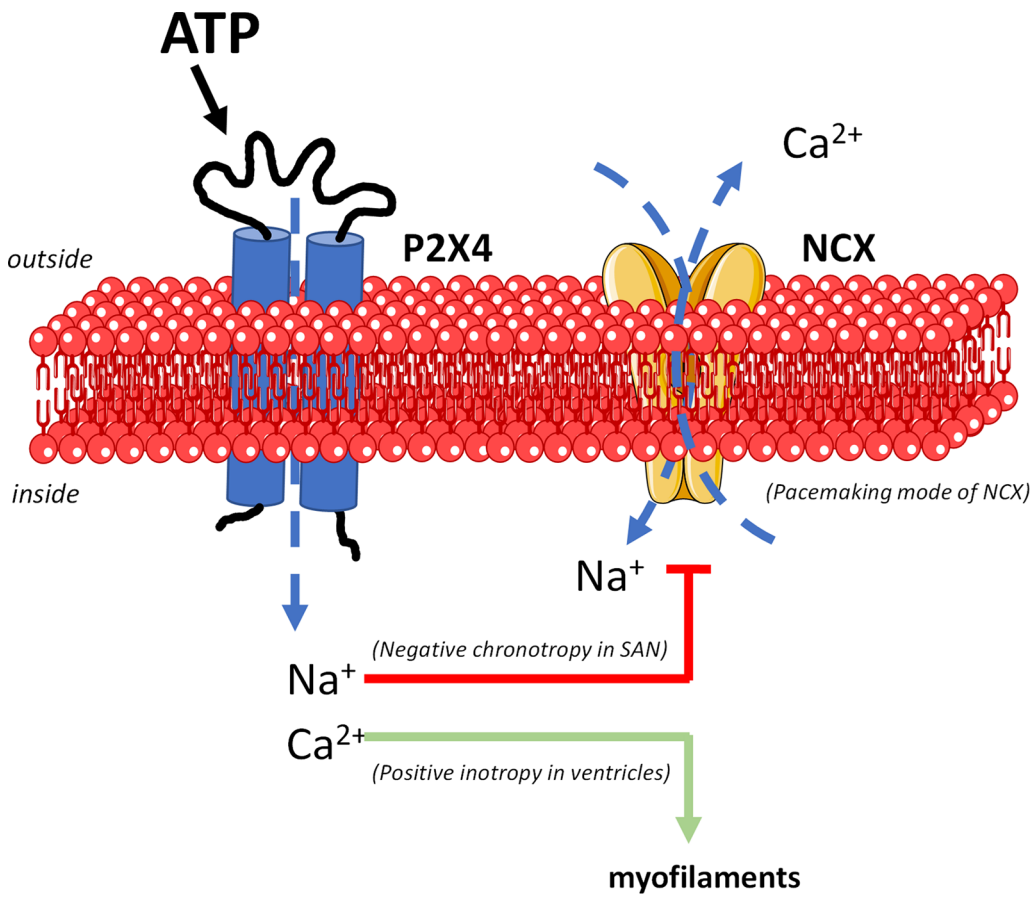

FIGURE 7 | The mechanism underlying the dual P2X4 receptor-mediated effects on cardiac chronotropy and inotropy implicates downstream modulation of NCX activity (digitalis-like phenomenon). Besides ion fluxes carried by pacemaker HCN channels (not represented), the unstable resting membrane potential and the spontaneous firing of SAN cardiomyocytes are attributed mainly to the electrogenic NCX transport operating in the forward Ca^{2+}-extrusion mode. Na^+ influx through the P2X4 receptor pore dissipates the electrochemical gradient of this ion across the plasma membrane leading to inhibition and/or reversion of the NCX pacemaker current. This may justify slowing down of SAN cells depolarizations and the negative chronotropic effect of ATP. Likewise, intracellular Ca^{2+} accumulation due both (1) to Ca^{2+} influx through the P2X4 receptor pore, and (2) to reversal of NCX activity may explain the positive inotropic effect of the P2X4 receptor in paced ventricular cardiomyocytes. Figure composition used elements from *Servier Medical Art*.

(Yegutkin, 2008; Headrick et al., 2013; Burnstock and Pelleg, 2015). Although the mammalian myocardium expresses multiple purinoceptors (Musa et al., 2009), the adenosine A_1 receptor has received most attention due to its relative abundance and well characterized functional role in the acute regulation of the heart (Musa et al., 2009; Chandrasekera et al., 2010; Headrick et al., 2013). Adenosine A_1 receptors activation decreases cardiac chronotropy, dromotropy, inotropy and counteracts adrenergic stimulation by a dual mechanism involving inhibition of adenylyl cyclase and opening of potassium channels (Belardinelli and Lerman, 1991; Burnstock and Pelleg, 2015; Braganca et al., 2016). Despite ATP effects may be mediated by breakdown to adenosine, the negative chronotropic action of ATP was insensitive to blockage of adenosine A_1 receptors with DPCPX used in a 6-fold higher concentration (3 nM) than that required to block this receptor (K_i~0.45 nM) (Lohse et al., 1987). However, one cannot exclude ATP conversion into adenosine during incubation with the nucleotide, yet even if this had occurred in our experimental conditions the amount of adenosine falls below the threshold to activate A_1 receptors in the SAN. These findings contrast with those obtained by Camara et al. (2015); these authors concluded that the negative chronotropic effect of ATP was dependent on A_1 receptors activation by using DPCPX in a concentration (1 μM) that is more than 2,000-fold higher than the K_i value for this antagonist to block the A_1 receptor (Camara et al., 2015). Under such conditions, off-target effects of DPCPX may appear, which include inhibition of phosphodiesterases that may explain reversal of the negative chronotropic effect of ATP (Camara et al., 2018).

Our theory that the negative chronotropic effect of ATP in spontaneously beating atria strips is mediated primarily *via* the activation of nucleotide-sensitive P2 purinoceptors is further supported by the fact that 1) it was reproduced by the enzymatically stable ATP analogue, ATPγS, 2) it was blocked by PPADS, a non-selective P2 purinoceptors antagonist exhibiting no affinity for adenosine receptors, and 3) prevention of ATP breakdown into adenosine with the NTPDase inhibitor, POM-1, increased rather than decreased ATP-induced negative chronotropism. Our findings agree with previous reports in the literature about the role of ATP and related adenine nucleotides on cardiac function (Versprille and van Duyn, 1966; Lundberg et al., 1984; Camara et al., 2015) and questions the most accepted hypothesis that the negative chronotropic action of ATP is most likely due to A_1 receptors activation after its rapid conversion into adenosine (Pelleg and Belhassen, 2010). While this hypothesis neglected the pivotal role of P2 purinoceptors in the control of spontaneous activity of the SAN, it has been demonstrated that ATP was more potent than adenosine in reducing heart rate (Pelleg et al., 1985; Sharma and Klein, 1988), which was interpreted as being due to an additional vagal reflex of ATP *via* sensory P2X2 and/or P2X3 receptors (Pelleg et al., 1987; Xu et al., 2005). This idea is difficult to admit in the present experimental conditions due to the fact that blockage of muscarinic acetylcholine receptors with atropine (1μM) failed to affect the rate and tension of spontaneous atrial contractions and did not modify ATP-induced effects, thus indicating that the cholinergic vagal tone is irrelevant for the P2-mediated effects of the nucleotide.

To the best of our knowledge, this is the first study demonstrating a role for the P2X4 receptor in the regulation of sinoatrial node automatism. Despite limited availability of selective drugs acting on the P2X4 receptor, it may be pharmacologically characterized by comparing agonists rank order of potency: ATP > 2-methylthioATP > CTP > α, β-methyleneATP (Soto et al., 1996), as well as by the use of selective antagonists and allosteric modulators (reviewed in Stokes et al., 2017). The potent and selective P2X4 receptor antagonist, 5-BDBD, with an IC_{50} value of about 1 μM, attenuated the negative chronotropic effect of ATP on spontaneously beating atria, while the positive allosteric modulator of the P2X4 receptor, ivermectin, potentiated the nucleotide response. Furthermore, we show here that besides ATP and its stable analogue, ATPγS, also the P2X4 agonist, CTP, whose hydrolysis does not directly yield adenosine, decreased the spontaneous atrial rate in a 5-BDBD-sensitive manner, but with a weaker potency comparing with adenine nucleotides (Soto et al., 1996; Kasuya et al., 2017). In this study we used 5-BDBD at a concentration (10 μM) that might also interfere with P2X1- and P2X3-mediated actions (Coddou et al., 2019), but we are confident that this is irrelevant in this case because very low amounts of these receptors are expressed in the SAN (Musa et al., 2009).

The P2X4 receptor shares structural and functional properties with other P2X receptors. For instance, it is known that the rat P2X4 receptor is relatively insensitive to PPADS (IC_{50}~100 μM), in contrast to mouse and human P2X4 orthologs (IC_{50}~10 μM; Jones et al., 2000). Although exhibiting a weaker potency for the rat P2X4 receptor, we almost prevented the negative chronotropic effect of ATP using 100 μM PPADS. One cannot, however, exclude a minor participation of PPADS-sensitive metabotropic P2Y receptors in the bradycardic effect of ATP. The ionotropic P2X4 receptor is slowly desensitized by ATP (Jarvis and Khakh, 2009). This feature might explain the relatively sustained negative chronotropic effect of ATP and its analogue, ATPγS, during the time (at least for 5 min) of incubation with these compounds. However, the sustained negative chronotropic effect of ATP does not explain the potentiating action the NTPDase inhibitor, POM-1, unless one hypothesizes that extracellular ATP accumulation also contributes to reduce adenosine formation by feed-forwardly inhibiting ecto-5'-nucleotidase/CD73, as demonstrated in other studies (Magalhães-Cardoso et al., 2003; Duarte-Araújo et al., 2009; Vieira et al., 2014).

Notwithstanding our observations, other studies failed to demonstrate the involvement of P2X receptors in the control of heart rate. For instance, infusion of 2-methylthioATP did not change heart rate in the Langendorff-perfused heart (Mei and Liang, 2001), most probably because the used concentration (100 nM) of the ATP analogue falls below the threshold (1 μM) required to activate the P2X4 in the SAN (Jarvis and Khakh, 2009). The same group also failed to find any difference in the spontaneous heart rate when comparing wild-type with mice overexpressing or missing the P2X4 receptor (Hu et al., 2001; Yang et al., 2014). One must, however, emphasize that these studies were designed to evaluate the P2X4 receptor tone under basal conditions, *i.e.* in the absence of any P2X4 agonist, which is a different situation from the present report. Thus, future studies are required to elucidate the role of the P2X4 receptor in the *in vivo* control of heart rate.

In the rat heart, the P2X4 receptor is the third most abundant P2X receptor after P2X7 and P2X5 receptors, while in the human heart it is considered the most expressed P2X receptor subtype (Musa et al., 2009). Also the regional distribution of the P2X4 receptor in the heart displays some differences among species. Using immunofluorescence confocal microscopy, we show here that the P2X4 protein is slightly more expressed in the plasma membrane of SAN cells (mostly cardiomyocytes, but also blood vessels and nerve fibers) followed by the RV and RA of the rat. This is slightly different from data obtained in humans where the P2X4 receptor mRNA seems to be evenly expressed through the myocardium (Musa et al., 2009).

P2X4-Induced Negative Chronotropism Requires Reversal of the NCX Activity Mode

Interestingly, the distribution of the P2X4 receptor in SAN cardiomyocytes matches the immunofluorescence staining pattern of NCX1 and HCN4 in the rat. This led us to hypothesize that the P2X4 receptor-mediated negative chronotropic effect of ATP could involve downstream modulation of NCX and/or HCN pacemaker activities. Crosstalk between P2X4- and NCX-mediated effects has been demonstrated (Shen et al., 2014). Opening of the P2X4 ion pore mediates the influx of positive charges, mainly Na^+ and Ca^{2+} in a 1:4 ratio (Jarvis and Khakh, 2009), in the proximity of NCX carriers, which might affect their operation mode. Indeed, the ATP analogue, 2-methylthioATP (3 μM), inhibited the electrogenic forward mode of NCX in ventricular myocytes *via* an increase (by about 1 mM) in the intracellular Na^+ concentration (Shen et al., 2014), which represents a net increase of 7–25% considering the resting intracellular Na^+ concentration (Despa and Bers, 2013). Increases in intracellular Na^+ may be even more relevant in cells with limited pathways for Na^+ entry due to low expression levels of voltage-sensitive Na^+ channels, like the SAN cardiomyocytes (Remme and Bezzina, 2010). Likewise, it has been demonstrated that persistent Na^+ currents evoked by veratridine triggers intracellular calcium transients by reversing the operation mode of NCX in CA1 pyramidal cells (Fekete et al., 2009). Although speculative, reversal of the NCX function mode by the influx of Na^+ represents an alternative mechanism for dysrhythmias (including bradycardia) in some inherited cardiac sodium channelopathies, such as the type 3 long QT syndrome associated with SCN5A mutations and persistent sodium currents (Remme and Bezzina, 2010). In agreement with our theory that Na^+ influx *via* the P2X4 receptor pore might affect the NCX mode of function to decrease heart rate (**Figure 7**), we showed here for the first time that partial blockage of NCX, but not HCN channel, with two distinct inhibitors, KR-R7943 or ORM-10103, turned the spontaneously beating atria less sensitive to the negative chronotropic effect of ATP. Although beyond the scope of the present work, the interplay between P2X4 and NCX deserves further investigations using highly-demanding electrophysiology patch-clamp techniques in acutely isolated SAN cardiomyocytes from both rats and humans (ongoing research project).

Co-localization of P2X4 and NCX1 immunoreactivity in NF160 positive neuronal fibers was also detected. The presence of the P2X4 receptor in neuronal structures is widely accepted, but its function remains to be explored (Stokes et al., 2017). The SAN and the surrounding myocardium are regulated by a dense network of autonomic fibers, which are mainly parasympathetic followed by a sympathetic origin (Crick et al., 1994; Crick et al., 1999; Pauza et al., 2013; Zarzoso et al., 2013; Rajendran et al., 2019). Interestingly, some intracardiac neurons within atria contain ATP stored in vesicles (Crowe and Burnstock, 1982), which upon activation may represent an important source of extracellular ATP (Burnstock, 1972; Fredholm et al., 1982; Tokunaga et al., 1995). Reversion of NCX forward activity during ischemic conditions contributes to increase the magnitude of Ca^{2+} transients and, thus, the release of neurotransmitters from presynaptic nerve terminals (Lee and Kim, 2015). Given the co-localization and putative interplay between the P2X4 receptor and NCX in NF160-positive nerve fibers, one may speculate that these players may also interact to control the activity of cardiac neurons (Griffioen et al., 2007). This is even more relevant taking into consideration that cardiac ischemia is accompanied by P2X4 overexpression, particularly in the SAN (Musa et al., 2009). Thus, ATP released from autonomic cardiac nerves may trigger a positive feedback loop involving the NCX leading to an increase in the purinergic control of atrial cardiomyocyte function at both pre- and post-junctional levels.

ATP-Induced Negative Inotropism Is Partially Offset by P2X4 Activation and NCX Transport Reversal

The inotropic effect of ATP was investigated in paced RV strips; the nucleotide decreased ventricular inotropy in a concentration-dependent manner, yet changes in paced ventricular tension were less potent than the recorded ATP-induced negative chronotropic actions in spontaneously beating atria. This raised the possibility for the existence of a yet unraveled ATP-induced negative inotropic offsetting mechanism. Although we did not fully characterized the receptors involved in the negative inotropic effect of ATP, previous studies agree that P2 purinoceptors activation may be necessary, also taking into consideration that adenosine plays a minor (if any) role on ventricular inotropy (Burnstock and Meghji, 1983; Belardinelli et al., 1995; Balogh et al., 2005). There is, however, a contention regarding to whether ATP exerts a positive or a negative inotropic effect on ventricular contractions. In contrast to our findings, most reports in the literature suggest that ATP exerts a predominant positive inotropic effect in the heart. Nonetheless, it is worth to emphasize that the vast majority of these studies were performed in isolated ventricular myocytes (Danziger et al., 1988; De Young and Scarpa, 1989; Christie et al., 1992; Podrasky et al., 1997; Mei and Liang, 2001; Balogh et al., 2005). These findings attenuate the theory that ATP-induced positive inotropism could be mediated by P2X4 receptors facilitating noradrenaline release from sympathetic nerve terminals in paced ventricular strips. Only three studies were performed in more complex tissue preparations, namely in rat papillary muscles (Legssyer et al., 1988; Scamps et al., 1990) and in the frog ventricle (Flitney and Singh, 1980). Interestingly, the

Legssyer's and Flitney's studies reported a dual and opposing role of ATP in cardiac tissues. In support of a negative inotropic role for ATP, a recent study performed in intact isolated hearts, as well as in ventricular fragments and acutely isolated myocytes proposed that diadenosine tetraphosphate decreased ventricular inotropy probably *via* the activation of P2Y purinoceptors (Pakhomov et al., 2018).

Notwithstanding the conflicting results regarding the nature of the inotropic role of ATP, the use of ATP analogues and more selective P2 receptor modulators, in combination with genetic and other advanced biochemical techniques, provided strong evidence that several P2X and P2Y receptors may be positive ventricular inotropic mediators (reviewed in Erlinge and Burnstock, 2008; Burnstock and Pelleg, 2015). Regarding the P2Y receptor family, positive inotropy is generally attributed to stimulation of Gs and Gq-protein coupled receptors (Erlinge and Burnstock, 2008). Among them, ATP preferentially activates the $P2Y_{11}$ receptor (Abbracchio et al., 2006). The selective $P2Y_{11}$ agonist, AR-C67085, increased contraction in isolated cardiomyocytes as well as in isolated trabecular preparations. In that study, $P2Y_{12}$ and $P2Y_{13}$ receptors were excluded by the lack of effect of the stable ADP analogue, 2-methylthioADP, in cardiomyocytes contractile activity, which also nearly exclude any involvement of ATP-sensitive Gi-protein coupled P2Y receptor (Balogh et al., 2005). Of note, it is likely that these authors performed their studies in a mixed population of ventricular and atrial cardiomyocytes, as these cells were not separated by the enzymatic digestion of the heart. The putative involvement of P2Y receptors in the negative inotropic effect of ATP in paced ventricular strips was not assessed here, which is a limitation of our study that certainly deserves further investigations along with the corresponding effects in the *in vivo* animal.

Given the involvement of the P2X4 receptor in the negative control of sinoatrial automatism (see above), we focused our interest at investigating the role of this ionotropic receptor on ventricular contractile activity (Erlinge and Burnstock, 2008; Burnstock and Pelleg, 2015). This question was raised because heart rate slowing drugs devoid of effect or with a moderate positive inotropic action on ventricular contraction may be relevant to treat heart failure. Our findings show that selective blockage of the P2X4 receptor activation with 5-BDBD significantly increased the negative inotropic effect of ATP in paced RV strips, thus suggesting that the P2X4 receptor may exert a counteracting positive inotropic action that is responsible for partially offsetting ATP-induce downsizing of ventricular contractions. As a matter of fact, the ATP analogues, 2-methylthioATP and α,β-methyleneATP, increased contractions of isolated ventricular cells, as well as of ventricular strips and isolated working hearts in rodents (Burnstock and Meghji, 1983; Hu et al., 2001; Mei and Liang, 2001). Overexpression of the P2X4 receptor 1) enhances ATP-induced cardiac contractility in the intact heart, and 2) rescues the systolic function and increase survival of animals with cardiomyopathy (Yang et al., 2004; Shen et al., 2009). The beneficial effects of the P2X4 receptor on cardiac function were attributed to activation of calcium-dependent endothelial-type nitric oxide synthase (Blaustein and Lederer, 1999; Yang et al., 2015). Ca^{2+} influx through the P2X4 receptor pore may itself account for the positive inotropic action of ATP analogues. On the other hand, Na^{+} influx through the P2X4 receptor also contributes to inhibit or, even revert, the electrogenic transport of NCX in the forward mode (Ca^{2+} extrusion mode) leading to an additional increase in the amplitude and duration of Ca^{2+} transients inside cardiomyocytes, which boosts their contractile activity (Shen et al., 2014; see **Figure 7**).

This concept may also explain the biphasic effect of ATP on atrial inotropy reported in this study and by other authors (Froldi et al., 1994; Gergs et al., 2008), which consisted of a transient decrease followed by a gradual recovery of the amplitude of atrial contractions while the preparations were still in contact with the nucleotide. Even though atrial inotropy represents an important reserve to maintain cardiac output in demanding conditions and in the setting of ventricular diastolic dysfunction, this phenomenon was not further evaluated in spontaneously beating rat atria due to significant bias introduced by changes in the rate of contractions.

Another relevant aspect of the P2X4 receptor regulation with potential implications for cardiac pathophysiology is its sensitivity to pH; in acidotic conditions, as it occurs in ischemia/hypoxia or renal failure, the P2X4 receptor activity significantly decreases, whereas the opposite occurs alkaline conditions (Wildman et al., 1999). Growing evidence exist demonstrating that the P2X4 receptor is overexpressed in ventricles under stressful conditions, namely in pulmonary hypertension and ischemia-induced heart failure (Sonin et al., 2008; Musa et al., 2009; Ohata et al., 2011). Altogether these findings strengthen the potential involvement of the P2X4 receptor in cardiac normal physiology and diseases progression.

CONCLUSION

Overall, data suggest that ATP-sensitive P2X4 ionotropic receptors play a major role in decreasing the spontaneous activity of the SAN while partially offsetting the negative inotropic effect of the nucleotide in paced rat ventricles. The mechanism underlying the dual P2X4 receptor-mediated effects on cardiac chronotropy and inotropy involves downstream interaction with the activity of NCX. Na^{+} influx *via* the P2X4 receptor pore may inhibit and/or revert the electrogenic forward current of the NCX, thus decreasing chronotropy. Likewise, intracellular Ca^{2+} accumulation due to interference with NCX might explain the positive inotropic effect attributed to the P2X4 receptor activation on paced RV strips (**Figure 7**). Regional differences observed for the distribution of the P2X4 receptor, along with its biophysical properties, bring new therapeutic opportunities for P2X4 activation with potential to create novel well-tolerated heart-rate lowering drugs with promising benefits in patients with deteriorated ventricular function.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The animal study was revised and approved by the competent national authority Direção Geral de Alimentação e Veterinária, and by the ICBAS Animal Ethical Committee (No. 224/2017).

AUTHOR CONTRIBUTIONS

PC-S supervised the project. BB and PC-S designed the experiments and wrote the manuscript. BB and SN-M carried out myographic recordings. BB and FF performed immunofluorescence confocal microscopy experiments. BB, FF, AF-S, and PC-S interpreted data, discussed the clinical implications, and commented on the manuscript at all stages.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01103/full#supplementary-material

FIGURE S1 | ATP (0.1 μM–1 mM) concentration-dependently decrease the spontaneous atrial rate. The negative chronotropic effect of ATP was mimicked by its enzymatically stable analogue, ATPγS (0.001–0.1 mM, A), and its potency was increased after pretreatment of the preparations with POM-1 (100 μM, a non-selective NTPDase inhibitor, B) and with ivermectin (30 μM, a positive allosteric modulator of the P2X4 receptor, C). For comparison purposes, we show in panel D that ATP (100 μM)-induced negative chronotropy was fully blocked by PPADS (100 μM). In panels A–C, data are expressed as mean ± SEM from an *n* number of animals. In panel D, represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians; each data point represents the result of a single experiment; data from the same experiment are connected by lines. *$p < 0.05$ (Student's *t*-test for paired samples) represent significant differences when compared to the effect of ATP alone.

FIGURE S2 | Effects of receptor antagonists/allosteric modulators, NTPDase inhibitor and ion transport blockers on spontaneously beating rat atria (A–J). POM-1 (100 μM, A), PPADS (10 μM, B; 100 μM, C), DPCPX (3 nM, D), A438079 (3 μM, E), 5-BDBD (10 μM, F), ivermectin (30 μM, G), KB-R7943 (3 μM, H), ORM-10103 (3 μM, I), and ZD7288 (300nM, J) contacted with the preparations at least for 15 min; the rate of spontaneous atrial contractions (beats min^{-1}) was measured immediately before ATP applications (see Figures 2–4) and compared to baseline conditions in the absence of any drug. Each data point represents the result of a single experiment; points from the same experiment are connected by lines. On the right hand-side of each panel, represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. *$p < 0.05$ (Student's *t*-test for paired samples) represent significant differences when compared to baseline.

FIGURE S3 | Effects of the P2X4 receptor antagonist, 5-BDBD (10 μM, A and B) and of the NCX inhibitor, KB-R7943 (3 μM, C and D) on paced right ventricular contractions. Drugs contacted with the preparations at least for 15 min; the amplitude of 2 Hz-paced ventricular contractions measured as active tension (mN/mg of wet tissue weight, panels A and C) and as the derivative of developed force over time (+dF/dt, mN/s, panels B and D) was measured immediately before ATP applications (see **Figure 5**) and compared to baseline conditions in the absence of any drug. Each data point represents the result of a single experiment; points from the same experiment are connected by lines. On the right hand-side of each panel, represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. *$p < 0.05$ (Student's *t*-test for paired samples) represent significant differences when compared to baseline.

FIGURE S4 | Representative confocal micrographs showing the immunolocalization of the P2X4 receptor (Apr-024, extracellular loop, Alomone) and of the HCN4 channel (Agp-004, Alomone) in the rat sinoatrial node (SAN); images obtained in right atria (RA) and right ventricle (RV) are also shown for comparison. It is worth noting that while the SAN was positive for both markers, RA and RV were positive for the P2X4 receptor (green) but negative for the HCN4 (magenta). Images were taken from one whole-mount preparation of the rat heart including the three regions, SAN, RA and RV. Dashed lines represent boundaries of the SAN region. White arrows indicate blood vessels including the SAN artery. Scale bar 30 μm.

FIGURE S5 | Representative confocal micrographs of SAN showing positive immunoreactivity against P2X4 (Apr-002, Alomone), NCX1 (Anx-011, Alomone), and NF160 (Ab7794, Abcam) proteins. The upper three panels show lower magnification confocal images of the SAN identified by the enrichment in NF160-positive nerve fibers; images also show surrounding atrial cardiomyocytes characterized as being NF160-negative and Cx43-positive. Images were taken from only one atrial preparation. Dashed lines represent boundaries of the SAN region. White arrows indicate blood vessels including the SAN artery. Scale bar 30 μm.

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MINI REVIEW
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Adenosine Triphosphate Release and P2 Receptor Signaling in Piezo1 Channel-Dependent Mechanoregulation

Linyu Wei[1†], Fatema Mousawi[2†], Dongliang Li[3], Sébastien Roger[4], Jing Li[5], Xuebin Yang[6] and Lin-Hua Jiang[1,2,4]*

[1] Sino-UK Joint Laboratory of Brain Function and Injury and Department of Physiology and Neurobiology, Xinxiang Medical University, Xinxiang, China, [2] School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds, United Kingdom, [3] Department of Physiology, Sanquan College of Xinxiang Medical University, Xinxiang, China, [4] EA4245, Transplantation, Immunology and Inflammation, Faculty of Medicine, University of Tours, Tours, France, [5] Lingnan Medical Research Centre, School of Medicine, Guangzhou University of Chinese Medicine, Guangzhou, China, [6] Department of Oral Biology, Faculty of Medicine and Health, University of Leeds, Leeds, United Kingdom

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Rosa Gomez-Villafuertes, Complutense University of Madrid, Spain

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***Correspondence:**
Lin-Hua Jiang
l.h.jiang@leeds.ac.uk

[†]These authors have contributed equally to this work

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Organs and tissues and their constituent cells are physiologically submitted to diverse types of mechanical forces or stress, one common sequence of which is release of intracellular ATP into extracellular space. Extracellular ATP is a well-established autocrine or paracrine signaling molecule that regulates multiple cell functions and mediates cell-to-cell communications *via* activating the purinergic P2 receptors, more specifically, ligand-gated ion channel P2X receptors and some of the G-protein-coupled P2Y receptors. The molecular mechanisms that sense mechanical and transduce forces to trigger ATP release are poorly understood. The Piezo1, a newly identified mechanosensing ion channel, shows widespread expression and confers mechanosensitivity in many different types of cells. In this mini-review, we briefly introduce the Piezo1 channel and discuss the evidence that supports its important role in the mechanoregulation of diverse cell functions and, more specifically, critical engagement of ATP release and subsequent P2 receptor activation in Piezo1 channel-dependent mechanoregulation. Such ATP release-mediated coupling of the Piezo1 channel and P2 receptors may serve a signaling mechanism that is more common than we currently understand in transducing mechanical information to regulation of the attendant cell functions in various organs and tissues.

Keywords: mechanical stimuli, mechanosensitive cells, Piezo1 channel, adenosine triphosphate release, P2 receptors

INTRODUCTION

Adenosine triphosphate (ATP), while it is best known for its intracellular role as the cellular energy source, gains increasing recognition as an extracellular signaling molecule when it is released into extracellular spaces. In mammalian cells, the ATP-based signaling system comprises of three principal components: release of intracellular ATP into the extracellular space, activation of the ligand-gated ion channel P2X receptors and/or G-protein-coupled P2Y receptors for extracellular ATP, and removal of extracellular ATP to terminate its action by a broad family of ATP-scavenging

ecto-nucleotidases that convert ATP to ADP, adenosine monophosphate, or adenosine (**Figure 1**) (Verkhratsky and Burnstock, 2014; Jiang et al., 2017a). This system represents one of the most common signaling mechanisms regulating cell functions and mediating cell-to-cell communications and plays a critical role in a wide range of physiological processes, such as hearing, tasting, nociception, immune responses, muscle contraction, learning, and memory. There exists a large volume of evidence that alterations in such an ATP-based signaling system contribute in the pathogenesis and progression of diverse conditions, ranging from hearing loss, pain, inflammatory diseases, hypertension, neurodegenerative diseases, and psychotic disorders to cancer metastasis (North, 2002; Fields and Burnstock, 2006; Khakh and North, 2006; Abbracchio et al., 2009; Surprenant and North, 2009; Zimmermann et al., 2012; Caseley et al., 2014; Roger et al., 2015; Cekic and Linden, 2016; Krugel, 2016; Alves et al., 2018; Di Virgilio et al., 2018; Wei et al., 2018).

It is conceivable that ATP easily leaks from damaged or dying cells as a danger signal alerting tissue damage and inflammation. However, decades of studies provide clear evidence to show that many types of cells can release ATP without compromise in cell viability and a variety of physical and chemical signals or stimuli can induce non-lytic release of ATP. Two general release pathways, namely, vesicular and diffusion, have been proposed for efflux of intracellular ATP (Verkhratsky and Burnstock, 2014). However, the molecular mechanisms mediating ATP release are still not fully elucidated, in part due to that such mechanisms appear to be diverse and cell-type specific. Furthermore, many types of cells are equipped with multiple ATP release mechanisms and deploy them according to the nature of the incoming stimuli. Vesicular release *via* exocytosis represents the major mechanism by which neurons release ATP into the synaptic cleft in the peripheral and central nervous systems (Pankratov et al., 2006; Abbracchio et al., 2009; Masuda et al., 2016). Vesicular ATP release *via* exocytosis has been also described in astrocytes (Chen et al., 2013; Lalo et al., 2014), urothelial cells (Nakagomi et al., 2016), neutrophils (Harada et al., 2018), and pancreatic β-cells (Geisler et al., 2013; Sakamoto et al., 2014). In this regard, it is worth mentioning that the vesicular nucleotide transporter (VNUT) plays a critical role in mediating vesicular storage and thereby subsequent release of ATP (Sawada et al., 2008) (for more details, see Moriyama et al., 2017; Miras-Portugal et al., 2019). On the other hand, several distinctive types of ion channels have been suggested to act as conduits permitting diffusion of ATP out of cells. The volume-regulated anion channel (VRAC) has been identified to mediate non-synaptic release of ATP from axons in response to action potential-induced swelling (Fields and Ni, 2010). The pannexin hemi-channels, calcium homeostasis modulator 1 (CALHM1), cystic fibrosis transmembrane conductance regulator (CFTR), maxi-anion channel, and P2X7 receptor as well as the VRAC have been reported to mediate or regulate ATP efflux from a variety of non-neuronal cells. For detailed discussion of these ATP release mechanisms, the readers can consult recently published reviews (e.g., Verkhratsky and Burnstock, 2014; Taruno, 2018).

Cells are physiologically submitted to diverse types of mechanical forces or stress and virtually all types of cells exhibit a mechanosensitivity. They can sense external or "outside-in" mechanical forces, for example, fluid flow-induced shear stress, osmotic stress, and pressure-induced membrane stretch (Nourse and Pathak, 2017). Cells can also generate traction forces *via* actin-myosin interactions at the focal adhesion zones and apply such "inside-out" mechanical forces to survey the mechanical and geographical properties of extracellular matrix and cell-supporting substrates (Nourse and Pathak, 2017; Ellefsen et al., 2019). Importantly, cells are able to convert mechanical forces into intracellular signals and even integrate mechanical information into the genomic blueprint (Choi et al., 2019), indicating that mechanical stimulation can have long-term effects as well as short-term effects on cell functions. Mechanical stimuli are long known as a potent trigger for non-cytolytic release of ATP both *in vivo* and *in vitro*, and accumulating evidence supports that

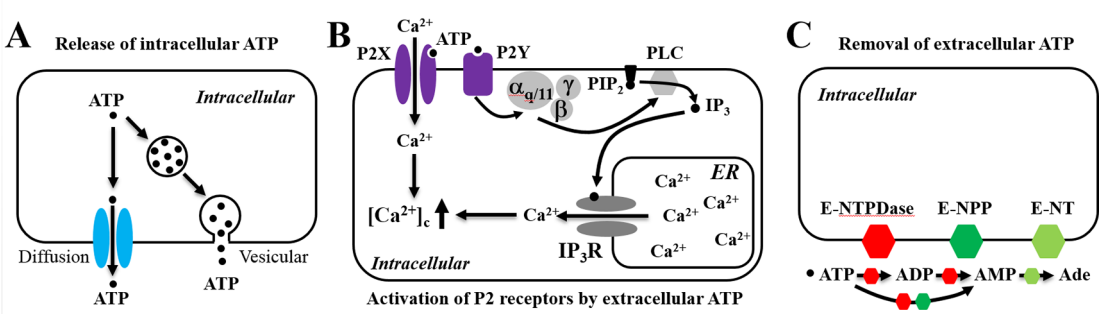

FIGURE 1 | Schematic illustration of the adenosine triphosphate (ATP)-based signaling system in mammalian cells. The ATP-based signaling system comprises of the following three principal components. **(A)** Release of intracellular ATP, which occurs *via* exocytosis (vesicular) and/or diffusion through many different types of ion channels. **(B)** Extracellular ATP as an autocrine or paracrine signal activating ligand-gated ion channel P2X receptors and/or G-protein-coupled P2Y receptors. ATP gates all P2X receptor ion channels, allowing extracellular Ca^{2+} influx. Alternatively, ATP activates the P2Y receptors, mainly $P2Y_1$, $P2Y_2$, and $P2Y_{11}$, leading to sequential activation of $G_{\alpha,q/11}$, phospholipase C (PLC), conversion of membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (not depicted), activation of the IP_3 receptor (IP_3R), and Ca^{2+} release from the endoplasmic reticulum (ER). **(C)** Termination of the actions of ATP by converting to ADP, AMP, and adenosine (Ade) by ecto-nucleosides, including ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), and by ecto-5'-nucelotidase (E-NT).

ATP release and subsequent activation of the P2 receptors act as a crucial signal transduction mechanism in the mechanoregulation of cell functions (Petruzzi et al., 1994; Riddle et al., 2007; Wan et al., 2008; Yu et al., 2010; Sun et al., 2013; Miyamoto et al., 2014; Weihs et al., 2014; Cinar et al., 2015; Wang et al., 2016; Iring et al., 2019). However, the molecular identity of the mechanosensor that directly detects the mechanical forces and triggers ATP release remained elusive. The Piezo1 ion channel has emerged as an intrinsically mechanically activated Ca^{2+}-permeable cation channel that confers cells with an ability of sensing diverse forms of mechanical stimuli (Murthy et al., 2017; Wu et al., 2017; Xiao, 2019). Furthermore, a large number of recent studies have shown an important role of the Piezo1 channel in the mechanoregulation of a wide range of physiological and pathological functions (Murthy et al., 2017; Wu et al., 2017; Xiao, 2019). Of interest, accumulating evidence supports that ATP release and P2 receptor signaling are important in mediating Piezo1 channel-dependent mechanoregulation. The two separate domains of investigation thus need to join forces in order to develop a full and mechanistic understanding of mechanoregulation. The aim of this mini-review is to introduce the Piezo1 channel and discuss the recent studies that provide evidence to support its crucial role in several types of mechanosensitive cells in the induction of ATP release and subsequent activation of the P2X or P2Y receptors and the mechanoregulation of the attendant cell functions. With increasing evidence to show their overlapping expression in many different types of mechanosensitive cells, the Piezo1 channel and P2 receptors, *via* coupling by ATP, may serve as a signaling mechanism that is more common than we currently understand in transducing the mechanical information to functional regulation.

A Brief Introduction to the Piezo1 Channel

The Piezo1 protein (also known as Fam38a) was identified to form the mechanically activated ion channel mediating pressure-induced ionic currents in mouse neuroblastoma Neuro2A cells (Coste et al., 2010; Coste et al., 2012; Syeda et al., 2016). In the same seminal study, a homologue protein, Piezo2 (also known as Fam38b), was found to express in a subset of mouse dorsal root ganglia neurons and can also form a mechanically activated ion channel with comparatively faster inactivation kinetics. The Piezo proteins are large in size, being ~2,500–2,800 amino acid residues long and with predicted molecular weights of ~290-320 kDa for the mouse and human proteins. They are predicted to have a unique membrane topology composed of 38 transmembrane segments and intracellular N- and C-termini (Zhao et al., 2019). Several structures containing the core parts of the mouse Piezo1 channel have been recently determined by cryo-electron microscopy (Saotome et al., 2018; Zhao et al., 2018; Wang et al., 2019). These structures reveal a trimeric assembly and a three-bladed propeller-like architecture of the Piezo1 channel. For further structural details, the readers can consult recently published reviews (e.g., Murthy et al., 2017; Xiao, 2019; Zhao et al., 2019).

Studies have demonstrated wide expression of the Piezo1 channel that enables many different types of cells to sense a diversity of "outside-in" mechanical forces, including indentation, membrane stretch, shear stress, osmotic stress, ultrasound, and compression (Coste et al., 2010; Li et al., 2014; Miyamoto et al., 2014; Pathak et al., 2014; Ranade et al., 2014; Jin et al., 2015; Lewis and Grandl, 2015; Syeda et al., 2016; Wang et al., 2016; Gao et al., 2017; Wu et al., 2017). There is also compelling evidence to suggest that the Piezo1 channel can be activated by traction forces (Pathak et al., 2014; Murthy et al., 2017; Nourse and Pathak, 2017; Ellefsen et al., 2019). Thus, two different, so-called "force-from-lipids" and "force-from-filaments," mechanisms have been proposed for mechanical activation of the Piezo1 channel (Murthy et al., 2017). In the "force-from-lipids" mechanism, mechanical forces introduce membrane tension that leads to reorganization of lipids within and surrounding the channel proteins. The resultant alterations in the membrane lipid-channel protein interactions induce the channel to open. This gating mechanism has gained support from a recent study (Lin et al., 2019). The "force-from-filaments" mechanism proposes that the interactions between the channel and extracellular matrix or intracellular cytoskeletal proteins provoke conformational changes leading to the channel opening.

The mechanically activated ion channels are less amenable to electrophysiological studies as compared to the ion channels activated by other modalities, such as changes in membrane potential, temperature, or chemical ligands. This is in part because of the unease of applying mechanical stimuli to cells under the experimental settings and the challenge of accurately determining the mechanical forces inducing the channel activation (Parpaite and Coste, 2017). Yoda1, a synthetic chemical, selectively activates the Piezo1 channel with an EC_{50} (the concentration evoking 50% of the maximal response) of 2.5–27 μM, determined by measuring Yoda1-induced Ca^{2+} responses in cells expressing the recombinant mouse and human Piezo1 channels (Syeda et al., 2015; Evans et al., 2018). The discovery of Yoda1 has made it technically more approachable to the study of the Piezo1 channel under *in vitro* conditions. Grammostola spatulata mechanotoxin 4 (GsMTx4), a 34-amino acid peptide isolated from the venom of a tarantula spider and known to block mechanically activated currents (Suchyna et al., 2000), has been shown to inhibit the Piezo1 channel in the low micromolar concentrations (Bae et al., 2011; Bagriantsev et al., 2014). Mechanistically, GsMTx4 acts on the extracellular side as a channel gating modifier to modulate the arrangements of membrane lipids in the surroundings of the channel protein and thereby decreases the efficiency of force transduction from the lipid bilayer to the channel (Suchyna et al., 2000; Gnanasambandam et al., 2017). Ruthenium red (RR), a polycationic ion, can also inhibit the Piezo1 channel-mediated mechanically activated currents with an IC_{50} (the concentration causing 50% inhibition of the response) of 5.4 μM, which was shown at the *Drosophila* Piezo channel, and RR is thought to be an open channel blocker (Coste et al., 2012). Gadolinium ion (Gd^{3+}) in the micromolar concentrations is known to inhibit the Piezo1 channel (Cinar et al., 2015). These negative allosteric modulators or inhibitors are lack of the specificity towards the Piezo1 channel (Bowman et al., 2007). Nonetheless, they provide useful pharmacological tools, in combination with genetic means, to better understand the role of the Piezo1 channel in physiological and pathological processes.

The expression of the Piezo1 channel has been shown in an increasing number of cell types in various tissues and organs,

including neurons, astrocytes, smooth muscle cells, endothelial cells, epithelial cells, red blood cells, immune cells, periodontal ligament cells, neural progenitor cells, mesenchymal stem cells, and embryonic stem cells (e.g., Li et al., 2014; Pathak et al., 2014; Jin et al., 2015; Gudipaty et al., 2017; Murthy et al., 2017; Del Marmol et al., 2018; Friedrich et al., 2019; Mousawi et al., 2019; Solis et al., 2019; Song et al., 2019; Velasco-Estevez et al., 2019). The Piezo1 channel is mainly located in the plasma membrane (Coste et al., 2010; Coste et al., 2012; Miyamoto et al., 2014; Hung et al., 2016). Some evidence suggests that the Piezo1 channel is also present in the membrane of endoplasmic reticulum (McHugh et al., 2010) and in the cytoplasmic compartments near the nucleus (Miyamoto et al., 2014) and nuclear envelope (Gudipaty et al., 2017). A large number of recent studies have disclosed a critical role for the cell surface Piezo1 channel or, more specifically, Piezo1-mediated Ca^{2+} influx, in the regulation of a multiple of cell functions (e.g., Li et al., 2014; Pathak et al., 2014; Cinar et al., 2015; Hung et al., 2016; Gudipaty et al., 2017; Del Marmol et al., 2018; Friedrich et al., 2019; Mousawi et al., 2019; Solis et al., 2019; Song et al., 2019; Velasco-Estevez et al., 2019; see a recent review by Xiao, 2019). As discussed next, accumulating evidence supports that ATP release and subsequent activation of the P2X and/or P2Y receptors are critical in mediating Piezo1 channel-dependent mechanoregulation.

Stretch-Induced Piezo1-Dependent Adenosine Triphosphate Release From Urothelial Cells and Regulation of Bladder Function

It is known that as the urinary bladder distends, the urothelial cells become stretched and, as a result, release ATP, which in turn excites the innervating pelvic nerve afferents (Ferguson et al., 1997;Vlaskovska et al., 2001; Beckel et al., 2015). The P2X3 receptor is expressed in the pelvic nerves, and the excitability of the pelvic nerve afferents induced by bladder distension was strongly attenuated in the P2X3-knockout mice, supporting a major role of the P2X3 receptor in transducing ATP release from urothelial cells to excitation of the pelvic nerve afferents (Vlaskovska et al., 2001). Both VNUT-dependent vesicular ATP release *via* exocytosis and ATP efflux through the CALHM1 and pannexin-1 hemi-channels have been shown to mediate ATP release from urothelial cells in response to mechanical forces (Beckel et al., 2015; Nakagomi et al., 2016; Sana-Ur-Rehman et al., 2017). Furthermore, transient receptor potential (TRP) channels, particularly the TRPV4 channel, were suggested to sense mechanical stretch to induce ATP release from urothelial cells (Mochizuki et al., 2009; Merrill et al., 2016). However, compelling evidence indicates that mechanical activation of the TRPV4 channel is indirect, depending on mechanical induction of phospholipase A2-mediated generation of arachidonic acid and/or P450 epoxygenase-mediated generation of 5′,6′-epoxyeicosatrienoic acid from arachidonic acid (Vriens et al., 2004; Berna-Erro et al., 2017). Thus, the molecular mechanism that directly senses mechanical stimuli to trigger ATP release from urothelial cells remained elusive. A recent study has shown expression of the Piezo1 channel in urothelial cells from both human and mouse bladders (Miyamoto et al., 2014). In addition, membrane stretch induced a Ca^{2+} influx-dependent increase in the $[Ca^{2+}]_i$ in mouse urothelial cells, and such Ca^{2+} response was strongly attenuated by treatment with GsMTx4 or small interference RNA (siRNA)-mediated knockdown of the Piezo1 expression. The same study has further found that stretch stimulated ATP release from mouse urothelial cells. Importantly, stretch-induced ATP release was dependent of extracellular Ca^{2+} and was suppressed by treatment with GsMTx4 or by siRNA-mediated knockdown of the Piezo1 expression. This recent study, taken together with the previous study identifying the P2X3 receptor in coupling urothelial ATP release to pelvic nerve afferent activation (Vlaskovska et al., 2001), supports the notion that the Piezo1 channel in urothelial cells sense the bladder distension and triggers ATP release from urothelial cells and that ATP in turn acts as a paracrine signal excites the pelvic nerve afferents *via* activation of the P2X3 receptor (**Figure 2A**). In other words, the Piezo1 channel in urothelial cells and P2X3

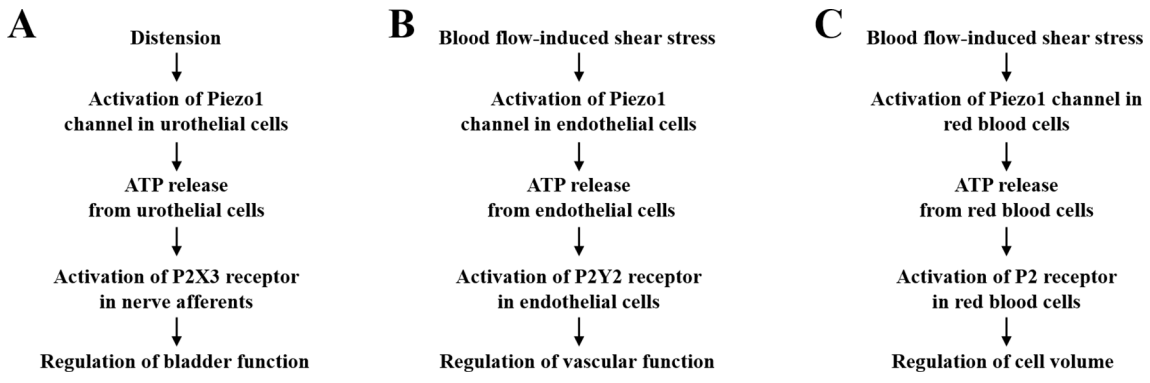

FIGURE 2 | Adenosine triphosphate (ATP) release and activation of P2 receptors in Piezo1 channel-dependent mechanoregulation. **(A)** The Piezo1 channel in urothelial cells senses mechanical forces resulting from bladder distension and induces urothelial cells to release ATP, which in turn acts as a paracrine signal to excite the pelvic nerve afferents by activating the P2X3 receptor. Such signaling mechanism is critical for maintaining the normal bladder function. **(B)** The Piezo1 channel in endothelial cells mediates blood flow-induced release of ATP that serves an autocrine signal acting on the P2Y2 receptor to regulate vascular function and blood pressure. **(C).** The Piezo1 channel in red blood cells mediates shear stress-induced release of ATP as an autocrine signal to activate yet identified P2 receptor(s) to regulate cell volume. See text for various molecular mechanisms that are known to mediate ATP release.

receptor in sensory neurons are important duo players, linked by ATP release from urothelial cells, to maintain the normal bladder function.

Shear Stress-Induced Piezo1-Dependent Adenosine Triphosphate Release From Endothelial Cells and Regulation of Vascular Function

The vascular endothelium experiences dynamic blood flow-induced shear stress. It is well recognized that the ability of endothelial cells to sense and respond to shear stress is vital for development, function, and disease of the vascular system (Hahn and Schwartz, 2009; Tarbell et al., 2014; Baeyens et al., 2016). ATP release from endothelial cells in response to shear stress has been well documented, and there is compelling evidence to support a critical role of the pannexin-1 hemi-channel in mediating shear stress-induced ATP release (Wang et al., 2015; Wang et al., 2016; Sathanoori et al., 2017). A recent study has shown that ATP released from endothelial cells upon exposure to shear stress serves as a paracrine signal that activates the P2Y2 receptor and downstream signaling pathways, including endothelial nitric oxide synthase to generate nitric oxide (NO), to induce vasodilation (Wang et al., 2015). Consistently, endothelium-specific deletion of the P2Y2 receptor expression in mice led to loss of blood flow-induced vasodilation, resulting in hypertension (Wang et al., 2015). A more recent study from the same group has examined the role of the Piezo1 channel in mediating shear stress-induced ATP release from endothelial cells (Wang et al., 2016). Exposing endothelial cells to shear stress or Yoda1 induced robust Ca^{2+} responses and ATP release, both of which were significantly attenuated by siRNA-mediated knockdown of the Piezo1 expression. ATP release induced by shear stress or Yoda1 was also suppressed by siRNA-mediated reduction in the expression of pannexin-1 or pannexin-2, indicating that shear stress-induced Piezo1-dependent ATP release is at least in part mediated by the pannexin hemi-channels (Wang et al., 2016). Perfusion of the mouse mesenteric arteries or exposure to Yoda1 induced vasodilation, which was impaired by endothelium-specific deletion of the Piezo1 expression. Furthermore, endothelium-specific and conditional knockout of the Piezo1 expression led to elevated blood pressure in mice (Wang et al., 2016), as observed for endothelium-specific and conditional knockout of the P2Y2 receptor (Wang et al., 2015). Collectively, these studies provide compelling evidence to support a vital role of the Piezo1 channel in mediating blood flow-induced release of ATP from endothelial cells as an autocrine signal to regulate the vascular function *via* activating the P2Y2 receptor (**Figure 2B**).

Shear Stress-Induced Piezo1-Dependent Adenosine Triphosphate Release From Red Blood Cells and Regulation of Cell Volume

Like endothelial cells, red blood cells in circulation are exposed to considerable flow-induced shear stress. Hereditary stomatocytosis and hereditary xerocytosis are rare genetic disorders characterized by red blood cell dehydration. Several gain-of-function mutations in the Piezo1 channel have been shown to be causatively associated these conditions, highlighting a crucial role of the Piezo1 channel in maintaining the normal red blood cell homeostasis (Zarychanski et al., 2012; Albuisson et al., 2013; Bae et al., 2013; Glogowska et al., 2017; Andolfo et al., 2018; Ma et al., 2018). Both human and mouse red blood cells are reported to express the Piezo1 channel on the cell surface. Interestingly, membrane stretch elicited strong Ca^{2+} influx-dependent increase in the $[Ca^{2+}]_i$ in red blood cells isolated from wild-type mice, but not from mice with conditional knockout of the Piezo1 expression (Cahalan et al., 2015). Similarly, exposure to Yoda1 induced Piezo1-dependent Ca^{2+} entry in mouse red blood cells (Cahalan et al., 2015). Fluid flow-induced shear stress also elicited robust Ca^{2+} influx in human red blood cells, which was significantly suppressed by treatment with GsMTx4, RR or Gd^{3+} (Cinar et al., 2015). Furthermore, genetic deletion of the Piezo1 expression led to red blood cell over-hydration and increased mechanical fragility both *in vitro* and *in vivo*. Conversely, Yoda1-induced activation of the Piezo1 channel caused red blood cell dehydration (Cahalan et al., 2015). These findings demonstrate an indispensable role of the Piezo1 channel in regulating red blood cell function and reveal the Piezo1 channel as a promising target for the development of therapeutics to treat hereditary stomatocytosis and hereditary xerocytosis.

It is long known that red blood cells release ATP in response to mechanical stimuli, such as osmotic stress (Petruzzi et al., 1994) and shear stress (Wan et al., 2008). A previous study showed that ATP release under *in vitro* conditions remained constant in response to shear stress below a certain threshold, but increased significantly above the threshold, which was accompanied with cellular deformation (Wan et al., 2008). A subsequent study provides evidence to suggest that the pannexin-1 hemi-channel is the main pathway mediating ATP release induced by shear stress both above and below the threshold, whereas the CFTR is engaged in deformation-dependent ATP release (Forsyth et al., 2011). A recent study shows that shear stress-induced ATP release was strongly correlated with extracellular Ca^{2+} concentration (Cinar et al., 2015). Shear stress-induced ATP release as well as Ca^{2+} influx in human red blood cells was attenuated by treatment with GsMTx4, RR or Gd^{3+} (Cinar et al., 2015). These results suggest that the Piezo1 channel is important in mediating induction by shear stress of ATP release from red blood cells (**Figure 2C**). Several ATP-sensitive P2 receptors, including P2X1, P2X7, and P2Y1, P2Y11 are expressed in red blood cells, and evidence exists to support that activation of these P2 receptors in red blood cells stimulates a number of signaling pathways that is critical for cell functions, including cell volume regulation (Sluyter, 2015). However, it has not been ascertained which P2 receptor(s) participate(s) in Piezo1-dependent regulation of red blood cell functions.

Piezo1-Dependent Adenosine Triphosphate Release From Mesenchymal Stem Cells and Regulation of Cell Migration

Mesenchymal stem cells (MSCs), which have promising applications in tissue regeneration and cell-based therapies, are highly mechanosensitive (Engler et al., 2006; Riddle et al.,

2007; Shih et al., 2011; Choi et al., 2012; Yang et al., 2012; Yuan et al., 2012; Suhr et al., 2013; Yuan et al., 2013; Chen et al., 2018; Goetzke et al., 2018; Li et al., 2018). It is well recognized that MSCs release ATP in response to mechanical stimulation both *in vitro* and *in vivo* (Riddle et al., 2007; Sun et al., 2013; Weihs et al., 2014). It is also known that several P2X and P2Y receptors are expressed in MSCs and mediate ATP-induced regulation of cell proliferation, migration, and differentiation (Coppi et al., 2007; Riddle et al., 2007; Sun et al., 2013; Peng et al., 2016; Jiang et al., 2017a; Jiang et al., 2017b). A previous study using bone marrow-derived MSCs suggests that fluid flow-induced ATP release *via* the pannexin hemi-channels and subsequent activation of the ATP-sensitive P2Y receptors increased cell proliferation (Riddle et al., 2007). A more recent study shows that shockwave-induced ATP release *via* undefined release mechanisms and subsequent activation of the P2X7 receptor stimulated osteogenic differentiation (Sun et al., 2013). The expression of the Piezo1 channel has been documented in several very recent studies using MSCs from different species and tissues (Gao et al., 2017; Sugimoto et al., 2017; Mousawi et al., 2019). Our recent study shows that Yoda1-induced activation of the Piezo1 channel in human dental pulp MSC promoted migration, which was suppressed by siRNA-mediated knockdown of the Piezo1 expression (Mousawi et al., 2019). More importantly, Yoda1-induced activation of the Piezo1 channel stimulated ATP release from human dental pulp MSCs (Mousawi et al., 2019). Yoda1-induced Piezo1-dependent increase in cell migration was inhibited by treatment with apyrase, a scavenger of extracellular ATP, and also with PPADS, a P2 receptor generic antagonist. Taken together, these results support the notion that activation of the Piezo1 channel enhances MSC migration *via* inducing release of ATP as an autocrine signal that activates the P2 receptors. Our previous study has identified P2X7, P2Y1, and P2Y11 as the major P2 receptors that participate in mediating ATP-induced stimulation of human dental pulp MSC migration (Peng et al., 2016). It is highly interesting to examine the role of ATP release and the P2 receptors in Piezo1-dependent mechanoregulation of MSC functions such as differentiation and migration.

Adenosine Triphosphate Release and P2 Receptor as a Common Signaling Mechanism in Piezo1 Channel-Dependent Mechanoregulation?

As mentioned above, recent studies demonstrate expression of the Piezo1 channel in many different types of mechanosensitive cells with an important role in the mechanoregulation of attendant cell functions. The majority, if not all, of these cells, are known to express the P2X/P2Y receptors that are important in mediating ATP-induced regulation of their functions. This raises the perspective that ATP release integrates the Piezo1 channel and P2 receptor as a more common signaling mechanism in the mechanoregulation of cell functions.

The expression of the Piezo1 channel is required for alignment of endothelial cells in response to shear stress (Li et al., 2014; Ranade et al., 2014). Similarly, the P2Y2 receptor in endothelial cells plays an important role in mediating shear stress-induced cell alignment (Sathanoori et al., 2017). It is interesting to investigate whether shear stress-induced ATP release couples the Piezo1 and the P2Y2 receptor in the regulation of vascular development. Another recent study shows that shear stress induces ATP release from red blood cells, on one hand, and an increase in the $[Ca^{2+}]_i$ and NO generation in endothelial cells and formation of inter-endothelial junctions, on the other. These shear stress-induced responses or effects both in red blood cells and endothelial cells were prevented by pharmacological inhibition and genetic depletion of the pannexin-1 channel on red blood cells (Xu et al., 2017). It is unknown whether shear stress-induced Piezo1-dependent ATP release from red blood cells acts as a paracrine signal to induce Ca^{2+} signaling in endothelial cells *via* activating the P2X/P2Y receptors.

As discussed above, ATP release coupling of the Piezo1 channel in urothelial cells and the P2X3 receptor in the pelvic nerve afferents is important in maintaining the normal bladder function. Such a signaling mechanism may also play an important role in mediating dentinal pain. It is known that dentinal fluid-induced odontoblast deformation can evoke dentinal pain. A recent electrophysiological study shows that pressure-induced odontoblast deformation elicited inward currents that caused membrane depolarization and induced action potentials in co-cultured isolectin IB4-negative medium-sized trigeminal ganglion neurons (Sato et al., 2018). Furthermore, such inward currents were significantly attenuated by treatment with NF110, a P2X3 receptor antagonist, or with GsMTx4 as well as with a cocktail of TRP channel inhibitors (Sato et al., 2018). It is thus hypothesized that Piezo1/TRP-dependent ATP release from odontoblasts in response to mechanical stimulation excites myelinated Aδ neurons *via* activating the P2X3 receptor, thereby forming a signaling mechanism generating dentinal pain.

Cancer cells in the metastasis process encounter mechanical forces such as compression from the surrounding extracellular matrix and cells in the primary site, invasion into neighboring tissues, intravasation and extravasation through endothelial cells, micro-metastasis at target tissues or organs. They also experience blood flow-induced shear stress during circulation in the blood stream. It is conceivable that mechanical forces influence cancer cell migration, invasiveness, and metastasis. Consistently, several recent studies provide increasing evidence to show that activation of the Piezo1 channel stimulates cell proliferation in gastric cancer cells (Zhang et al., 2018), and enhances cell migration in gastric cancer cells (Yang et al., 2014; Zhang et al., 2018) and malignant MCF-7 breast cancer cells (Li et al., 2015) but reduces non-small cell lung cancer progression and cell migration (Huang et al., 2019). Compelling evidence already exists to support that extracellular ATP can regulate cancer cell migration, invasiveness, and metastasis *via* activating the P2X7, P2Y2 or P2Y11 receptors (Jelassi et al., 2011; Jelassi et al., 2013; Schumacher et al., 2013; Chadet et al., 2014; Roger et al., 2015; Khalid et al., 2017). Particularly, it was shown that ATP released from platelets bound to the circulating cancer cells and consequently activates the P2Y2 receptor on endothelial cells to promote formation of inter-endothelial junctions for cancer cell migration (Schumacher et al., 2013). As discussed above, shear stress can induce ATP release from red blood cells. It is

attractive to speculate that shear stress-induced ATP release from red blood cells acts as a paracrine signal to induce formation of inter-endothelial junctions *via* activating the P2X/P2Y receptors in endothelial cells and thereby facilities intravasation and extravasation of cancer cells.

CONCLUDING REMARKS

It is evident from the discussion above that accumulating evidence supports an important role of ATP release as an autocrine and/or paracrine signal and subsequent activation of the P2 receptors in Piezo1 channel-dependent mechanoregulation of cell functions and associated physiological processes (**Figure 2**). As illustrated by hereditary stomatocytosis and hereditary xerocytosis, alterations in such signaling mechanisms resulting from mutations in the Piezo1 channel in red blood cells can lead to cell dysfunction and severe human diseased conditions. Studies so far support the Piezo1 channel as an intrinsic mechanosensor to trigger ATP release in response to mechanical stimulation. However, it remains unknown how activation of the Piezo1 channel regulates the ATP release mechanisms. Finally, more investigations are required to determine whether the Piezo1 channel and P2 receptors coupled by ATP release form a common signaling mechanism in transducing mechanical force information to regulation of cell functions.

AUTHOR CONTRIBUTIONS

All authors contributed to the development of the concept. L-HJ wrote the manuscript. All the authors commented and approved the manuscript.

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Differential Expression of the Metabotropic P2Y Receptor Family in the Cortex Following Status Epilepticus and Neuroprotection *via* $P2Y_1$ Antagonism in Mice

Mariana Alves[1†], *Jonathon Smith*[1,2†] *and Tobias Engel*[1,2*]

[1] Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland, [2] FutureNeuro SFI Research Centre, Dublin, Ireland

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***Correspondence:**
Tobias Engel
tengel@rcsi.ie

[†]*These authors have contributed equally to this work*

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Purinergic signaling *via* P2 receptors is now widely accepted to play a critical role during increased states of hyperexcitability and seizure-induced pathology. In the setting of seizures and epilepsy, most attention has been paid to investigating the fast-acting ATP-gated P2X receptor family. More recent evidence has now also provided compelling evidence of an involvement of the slower-acting P2Y receptor family during seizures. This includes data demonstrating expression changes of P2Y receptors in the hippocampus following acute seizures and during epilepsy and anticonvulsive properties of P2Y-targeting drugs; in particular drugs targeting the $P2Y_1$ subtype. Seizures, however, also involve damage to extra-hippocampal brain regions such as the cortex, which is thought to contribute to the epileptic phenotype. To analyze expressional changes of the P2Y receptor family in the cortex following status epilepticus and to determine the impact of drugs interfering with $P2Y_1$ signaling on cortical damage, we used a unilateral mouse model of intraamygdala kainic acid-induced status epilepticus. Analysis of cortical tissue showed that status epilepticus leads to a global up-regulation of the P2Y receptor family in the cortex including $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$, with the $P2Y_1$ and $P2Y_4$ receptor subtypes showing the strongest increase. Supporting a detrimental role of $P2Y_1$ activation during status epilepticus, treatment with the $P2Y_1$ agonist MRS2365 exacerbated high frequency high amplitude spiking, synonymous with injury-causing electrographic activity, and treatment with the $P2Y_1$ antagonists MRS2500 protected against seizure-induced cortical damage. Suggesting $P2Y_1$-mediated effects are predominantly due to increased microglia activation, treatment with the broad-spectrum anti-inflammatory drug minocycline abolished the observed neuroprotective effects of $P2Y_1$ antagonism. In conclusion, our results further support a role for $P2Y_1$-mediated signaling during seizure generation and seizure-induced neurodegeneration, suggesting $P2Y_1$-targeting therapies as novel treatment for drug-refractory status epilepticus.

Keywords: adenosine triphosphate, purinergic signaling, metabotropic P2 receptor family, status epilepticus, neurodegeneration, cortex

INTRODUCTION

Epilepsy is characterized by an enduring predisposition to increased hyperexcitability states in the brain and is one of most common chronic brain diseases affecting up to 70 million people worldwide. Despite the increasing number of anti-epileptic drugs available in the clinic, drug resistance to pharmacological interventions remains steadily at 30% (Thijs et al., 2019). Status epilepticus, medical emergency defined as prolonged continuous seizure activity lasting longer than 5 min, is associated with high mortality and can cause wide-spread brain damage and serious neurological complications including the development of epilepsy (Betjemann and Lowenstein, 2015). As for epilepsy, drug refractoriness during status epilepticus remains equally high, with patients not responding to treatment being particularly vulnerable to adverse clinical outcomes (Novy et al., 2010). Mounting data has demonstrated an important role for neuroinflammation during both seizure generation and epileptogenesis (Vezzani et al., 2016); consequently, current research in epilepsy has a strong focus on the identification of the molecular mechanisms responsible for driving inflammatory processes during seizure-induced pathology.

Purinergic signaling *via* extracellular adenine and uracil nucleotide-activated P2 receptors has been suggested as possible link between neuroinflammation and increased hyperexcitability states (Henshall and Engel, 2015; Rassendren and Audinat, 2016; Alves et al., 2018). P2 receptors are subdivided into the fast-acting P2X receptor family, activated mainly by adenosine tri-phosphate (ATP) and consisting of seven members (P2X1-7) and the slower acting metabotropic P2Y receptor family, activated by ATP, adenosine di-phosphate (ADP) and the uracil nucleotides uracil tri-phosphate (UTP), uracil di-phosphate, and UTP-glucose consisting of eight members ($P2Y_{1,2,4,6,11,12,13,14}$). P2Y receptors are further subdivided into groups based on their coupling to specific G proteins with $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ coupled to Gq proteins, ultimately resulting in the activation of protein kinase C *via* release of Ca^{2+} from intracellular stores. Among these, $P2Y_{11}$ can also couple to Gs. $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ are coupled to Gi proteins decreasing cAMP production *via* inhibiting adenylate cyclase (von Kugelgen, 2006). Both P2X and P2Y receptors are distributed widely throughout the central nervous system where they are expressed and are functional on many cell types, including neurons, microglia, astrocytes, and oligodendrocytes (Burnstock, 2007).

Mounting data has repeatedly demonstrated distinct changes in the expression profile of P2X and P2Y family members following acute seizures and during epilepsy, and provided compelling evidence that drugs blocking P2X or P2Y members alter seizure severity and may even impact on the development of epilepsy (Amhaoul et al., 2016; Engel et al., 2016; Amorim et al., 2017; Alves et al., 2018). While most efforts have been invested to study the effects of the fast-acting P2X receptor family on seizures and epilepsy, in particular the P2X7 receptor (Beamer et al., 2017), increasing evidence also suggest a causal role for P2Y receptors during seizure-induced pathology (Eyo et al., 2014; Avignone et al., 2015; Alves et al., 2017; Alves et al., 2018; Alves et al., 2019). P2Y receptor expression is altered in the hippocampus following status epilepticus and during epilepsy (Alves et al., 2017) and ADP and UTP, both broad-spectrum P2Y receptor agonists, alter seizure severity during status epilepticus and seizure-induced neurodegeneration (Alves et al., 2017). Further supporting a role for P2Y receptors during seizures, mice deficient in $P2Y_{12}$ display a more severe seizure phenotype during status epilepticus (Eyo et al., 2014). $P2Y_1$ antagonism has been shown to reduce seizure severity and protect against hippocampal neurodegeneration (Simoes et al., 2018; Alves et al., 2019). This is of no surprise considering the well documented role of $P2Y_1$ to regulate neurotransmitter release, facilitate neuronal excitability, and mediate microglia migration and activation (Guzman and Gerevich, 2016). Moreover, slice work in hippocampal tissue from epileptic rats showed that $P2Y_1$ antagonism caused a reduction in astrocytic Ca^{2+}-dependent glutamate gliotransmission and in turn hyperexcitability (Wellmann et al., 2018). Providing more evidence of an involvement of $P2Y_1$ during seizure generation, $P2Y_1$ antagonism decreases tumor necrosis factor-α–induced glutamate release from astrocytes and restores synaptic activity in hippocampal slices from epileptic mice (Nikolic et al., 2018).

To date, the investigation of P2Y expression and function has been mainly restricted to the hippocampus. Status epilepticus, however, also leads to cell death in extrahippocampal brain tissues including the amygdala, and piriform and entorhinal cortex in both experimental models of status epilepticus and in humans (Fujikawa et al., 2000; Curia et al., 2008; Mouri et al., 2008; Kienzler et al., 2009), which is thought to contribute to cognitive deficits and lowering of the seizure threshold (Thompson and Duncan, 2005; Helmstaedter, 2007). To establish whether P2Y signaling is involved in extrahippocampal neurodegeneration during status epilepticus, we characterized the expression profile of the P2Y receptor family in the cortex following status epilepticus and evaluated whether drugs targeting the $P2Y_1$ receptor subtype protect the cortex from seizure-induced damage (Mouri et al., 2008).

MATERIALS AND METHODS

Intraamygdala Kainic Acid Mouse Model of Status Epilepticus

All animal experiments were performed in accordance with the principles of the European Communities Council Directive (2010/63/EU). Procedures were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (REC 1322) and HPRA (AE19127/P038; AE19127/P001) and undertaken as described before (Engel et al., 2012). Experiments were carried out using 8- to 12-week-old C57Bl/6 male mice bred at the Biomedical Research Facility at RCSI and male *$P2Y_1$ knock-out* (KO) mice obtained from The Jackson Laboratory (009131-B6.129P2-P2ry1 < tm1Bhk>/J). Animals were housed in a controlled biomedical facility on a 12-h light/dark cycle at 22 ± 1°C and humidity of 40% to 60% with food and water provided ad libitum. During stereotaxic procedures, mice

were anesthetized using isoflurane (5% induction, 1%–2% maintenance) and maintained normothermic by means of a feedback-controlled heat blanket (Harved Apparatus Ltd, Kent, UK). Once fully anesthetized, mice were placed in a stereotaxic frame and a midline scalp incision was performed to expose the skull. A guide cannula (coordinates from Bregma; AP = −0.94 mm, L = −2.85 mm) and three electrodes for EEG recording (Bilaney Consultants, Sevenoaks, UK), two above each hippocampus and one above the frontal cortex as reference, were fixed in place with dental cement. EEG was recorded using the Xltek recording system (Optima Medical, Guildford, UK). Status epilepticus was induced by a microinjection of 0.3 μg kainic acid (KA) [0.2 μl phosphate-buffered saline (PBS)] (Sigma-Aldrich, Dublin, Ireland) into the right basolateral amygdala. Vehicle-injected control animals received 0.2 μl of PBS. The anticonvulsive lorazepam (6 mg/kg) (Wyetch, Taplow, UK) was delivered i.p. 40 min following intraamygdala KA or vehicle to curtail seizures and reduce morbidity and mortality.

Drug Administration

Mice were assigned randomly to receive either vehicle (sterile H_2O), $P2Y_1$ antagonist MRS2500 (MRS25) (1 nmol) (≥96% purity; Tocris Bioscience, Abingdon, UK) or $P2Y_1$ agonist MRS2365 (MRS23) (1 nmol) (98% purity; Tocris Bioscience, Abingdon, UK) 15 min following intraamygdala KA injection. All drugs were delivered by an intracerebroventricular (i.c.v.) microinjection (2 μl) into the ipsilateral lateral ventricle (coordinates from Bregma: AP = −0.4 mm; L = −0.95 mm). Minocycline (30 mg/kg, PBS) (Sigma-Aldrich (M9511), Dublin, Ireland) was administered twice *via* i.p. injection (200 μl) 24 and 4 h before triggering status epilepticus *via* intraamygdala KA (Alves et al., 2019).

EEG Analysis

The duration of high-frequency (> 5 Hz) and high-amplitude (> 2 times baseline) polyspike discharges of ≥5 s duration, synonymous with injury-causing electrographic activity (Araki et al., 2002), was counted manually by a reviewer unaware of treatment as before (Engel et al., 2012).

Western Blotting

To analyze expression changes of the P2Y receptor family in cortical tissue post-status epilepticus, the entire ipsilateral cortex was removed and homogenized in lysis buffer, and 30 μg of protein samples were loaded into an acrylamide gel and separated by SDS-PAGE electrophoresis. Membranes were probed with antibodies against $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (Alomone Labs, Hadassah Ein Kerem, Jerusalem, Israel), c-Fos (Santa Cruz, Heidelberg, Germany), and β-actin (Sigma-Aldrich, Dublin, Ireland). Protein bands were visualized using a Fujifilm LAS-4000 system (Fujifilm, Tokyo, Japan) with chemiluminescence (Pierre Biotechnology, Rockford, IL, U.S.A.), which was followed by analysis using Alpha-EaseFC4.0 software.

Fluoro-Jade B

Status epilepticus-induced neuronal cell death was assessed by Fluoro-Jade B (FjB) as described before (Engel et al., 2012). Twelve-micrometer coronal sections at the medial level of the hippocampus (Bregma AP = −1.94 mm) were sliced on a cryostat. Brain tissue was then fixed in 4% paraformaldehyde (PFA), rehydrated in ethanol, and transferred to a 0.006% potassium permanganate solution. Tissue sections were incubated with 0.001% FjB (Chemicon Europe Ltd, Chandlers Ford, UK) and mounted in dibutylphthalate polystyrene xylene mounting solution. Using an epifluorescence microscope, FjB-positive cells were counted under a 40× lens in two adjacent sections and the average determined for each animal.

Immunofluorescence Staining

To perform immunofluorescence staining, mice were anaesthetized with an overdose of 250 μl sodium pentobarbital (200 mg/ml) delivered i.p. and transcardially perfused with 4% PFA. Brains were then transferred to a solution of PBS and immersed into a 4% agarose solution before sectioning in a VT1000S vibratome. Thirty-micrometer brain sections were incubated in 0.1% Triton X-100 and glycine followed by the blocking solution (1% BSA-PBS). Brain tissue was then incubated with the primary antibodies: $P2Y_1$ (1:100) (Santa Cruz, Heidelberg, Germany), NeuN (1:400) (Millipore, Billerica, MA, U.S.A), GFAP (1:400) (Sigma-Aldrich, Dublin, Ireland), S100β (1:400) (Synaptic Systems, Goettingen, Germany), or Iba1 (1:400) (Wako, Neuss, Germany). Brain tissue was washed and incubated with a secondary antibody raised in goat conjugated with Alexa Fluor 488 and Alexa Fluor 568. Sections were stained with DAPI (1:500) and mounted onto glass slides with FluoroSave reagent. Confocal images were taken on a Zeiss Examiner Z1 microscope using a 40× immersion oil objective (Leica Microsystems, Wetzlar, Germany). Each image depicted in the results section is a representative picture from at least three mice. To determine the total number of $P2Y_1$-positive NeuN and Iba1 cells, three images from the cortical layer V–VI were obtained using a 40× lens in the Zeiss Examiner Z1 confocal microscope. Cell counts were the result of the average counting of images and were carried out unaware of treatment groups.

Statistical Analysis

For statistical analysis we used GraphPad Prism and STATVIEW software. Data was presented as means ± standard error of the mean. One-way analysis of variance with *post hoc* Fisher's protected least significant difference test was used to analyze three or more group data. For two-group comparison, Student's t-test was used to determine statistical differences between groups. Significance was accepted at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

P2Y Expression Changes in the Cortex Following Status Epilepticus

To determine status epilepticus-induced changes in the expression profile of the P2Y receptor family in cortical tissue, we used the intraamygdala KA mouse model of status epilepticus

(Araki et al., 2002; Mouri et al., 2008). In this model, status epilepticus leads to a characteristic lesion restricted to the ipsilateral brain hemisphere including the hippocampus and the cortex. While in the hippocampus cell death is mainly observed in the CA3 subfield, within cortical structures, neurodegeneration is most prominent in the cortical layers V and VI (**Figure 1A**) (Mouri et al., 2008). Increased levels of the activity-regulated protein c-Fos at 8 h following status epilepticus confirmed the recruitment of the ipsilateral cortex during status epilepticus (**Figure 1B**).

Previous work by us using the intraamygdala KA mouse model of status epilepticus has shown a distinct expression profile of the P2Y receptor family following status epilepticus (Alves et al., 2017). To determine whether status epilepticus also impacts on the expression of the P2Y receptor family in the cortex, tissue from the ipsilateral cortex was analyzed at different time-points post-status epilepticus *via* Western blot. This revealed an increase in the expression of several P2Y receptor family members including $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ (**Figure 1C**). While $P2Y_1$, $P2Y_2$, and $P2Y_6$ showed a significant increase at 24 h post-status epilepticus, $P2Y_4$ expression was already increased 1 h following status epilepticus and remained increased for up to 24 h (**Figure 1C**). Although displaying a slight increase in their expression, no significant expression changes could be observed for the remaining P2Y receptors post-status epilepticus (**Figure 1C**). $P2Y_{11}$ expression was not analyzed due to the lack of a *P2ry*$_{11}$ gene ortholog in the mouse genome (Dreisig and Kornum, 2016).

Taken together, our results demonstrate changes in the expression of P2Y receptors in the cortex following status epilepticus with P2Y upregulation being the predominant response.

Increased $P2Y_1$ Expression in Microglia Following Status Epilepticus

Emerging evidence suggests a causative role for P2Y signaling during seizure generation and seizure-induced pathology (Alves et al., 2018). Among the P2Y receptors analyzed, $P2Y_1$ was one of the receptors showing the strongest increase in its expression in the cortex following status epilepticus. Importantly, recent data suggests a functional role of $P2Y_1$ during status epilepticus and epilepsy (Alves et al., 2019). Therefore, to test whether P2Y receptor activation impacts on cortical neurodegeneration, our subsequent studies focused on the $P2Y_1$ receptor subtype.

First, to explore the cell-specific expression pattern of $P2Y_1$ in the cortex post-status epilepticus, we carried out co-immunostainings using different cell-type markers including NeuN for neurons, Iba1 for microglia, and GFAP and S100β for astrocytes and analyzed cortical layer V and VI, the areas were we observed most neuronal damage post-status epilepticus. Cortical tissue was analyzed 24 h following status epilepticus, the peak of $P2Y_1$ post-status epilepticus expression in the cortex. While $P2Y_1$ was detectable at low levels in cortical neurons and microglia under control conditions and following status epilepticus (**Figures 2A, B**), no co-localization was observed using the astrocyte markers GFAP and S100β in vehicle-injected control mice and in mice subjected to status epilepticus (**Figures 2C, D**). In line with previous results analyzing hippocampal $P2Y_1$ expression (Alves et al., 2019), $P2Y_1$ staining, however, strongly increased on Iba1-positive microglia 24 h post status epilepticus (**Figure 2E**). Higher magnification showed that, whereas cortical neuronal $P2Y_1$ seemed to be localized to the cell body with a punctate expression pattern, microglia $P2Y_1$ expression was observed throughout the cell including microglia processes (**Figure 2F**). Specificity of $P2Y_1$ staining was confirmed using brain tissue from $P2Y_1$ knock-out mice subjected to intraamygdala KA-induced status epilepticus (**Figure 2G**).

Thus, as observed previously in the hippocampus, status epilepticus leads to a strong increase in $P2Y_1$ immunoreactivity on microglia in the cortex.

$P2Y_1$ Antagonism Decreases High Frequency High Amplitude Spiking During Status Epilepticus

High frequency high amplitude (HFHA) spiking during status epilepticus has been shown to correlate with cell death in the intraamygdala KA mouse model (Araki et al., 2002). We have previously shown that treatment with the $P2Y_1$ agonists MRS2365 (MRS23) during status epilepticus increased total seizure power and treatment with the $P2Y_1$ antagonists MRS2500 (MRS25) reduced total seizure power (Alves et al., 2019). To determine whether targeting of $P2Y_1$ also impacts on HFHA spiking and thereby potentially on seizure-induced neurodegeneration, we re-analyzed EEG traces and quantified HFHA spiking in mice subjected to intraamygdala KA and treated with the $P2Y_1$ agonist MRS23 or $P2Y_1$ antagonist MRS25 15 min following the induction of status epilepticus (Alves et al., 2019). This revealed that mice treated with the $P2Y_1$ agonist MRS23 displayed a significant increase in HFHA spiking when compared to vehicle-treated mice (**Figures 3A, B**). No significant effect could be observed in mice treated with the $P2Y_1$ antagonist MRS25 when compared to vehicle-injected mice, although MRS25-treated mice showed a ~40% reduction in HFHA spiking when compared to control (Veh (527.7 ± 85.61 s) vs. MRS25 (317.7 ± 38.57 s), $p = 0.0542$) (**Figures 3A, B**). To test whether effects of $P2Y_1$ are mediated *via* inflammation, mice were treated with the broad-spectrum anti-inflammatory drug minocycline (Abraham et al., 2012; Alves et al., 2019). In line with anti-convulsive effects of $P2Y_1$ antagonism being mediated *via* inflammation, mice pre-treated with minocycline and injected with the $P2Y_1$ antagonist MRS25 showed no seizure reduction when compared to vehicle-injected mice subjected to intraamygdala KA status epilepticus and pre-treated with minocycline (**Figure 3C**).

In summary, $P2Y_1$ activation contributes to HFHA spiking during status epilepticus possibly mediated *via* driving pro-inflammatory processes in the brain.

$P2Y_1$ Antagonism Protects the Cortex From Seizure-Induced Neurodegeneration

Several studies have reported $P2Y_1$ antagonism to be protective against seizure-induced neurodegeneration in the hippocampus (Simoes et al., 2018; Alves et al., 2019). To determine whether $P2Y_1$ antagonism also protects against cortical cell death,

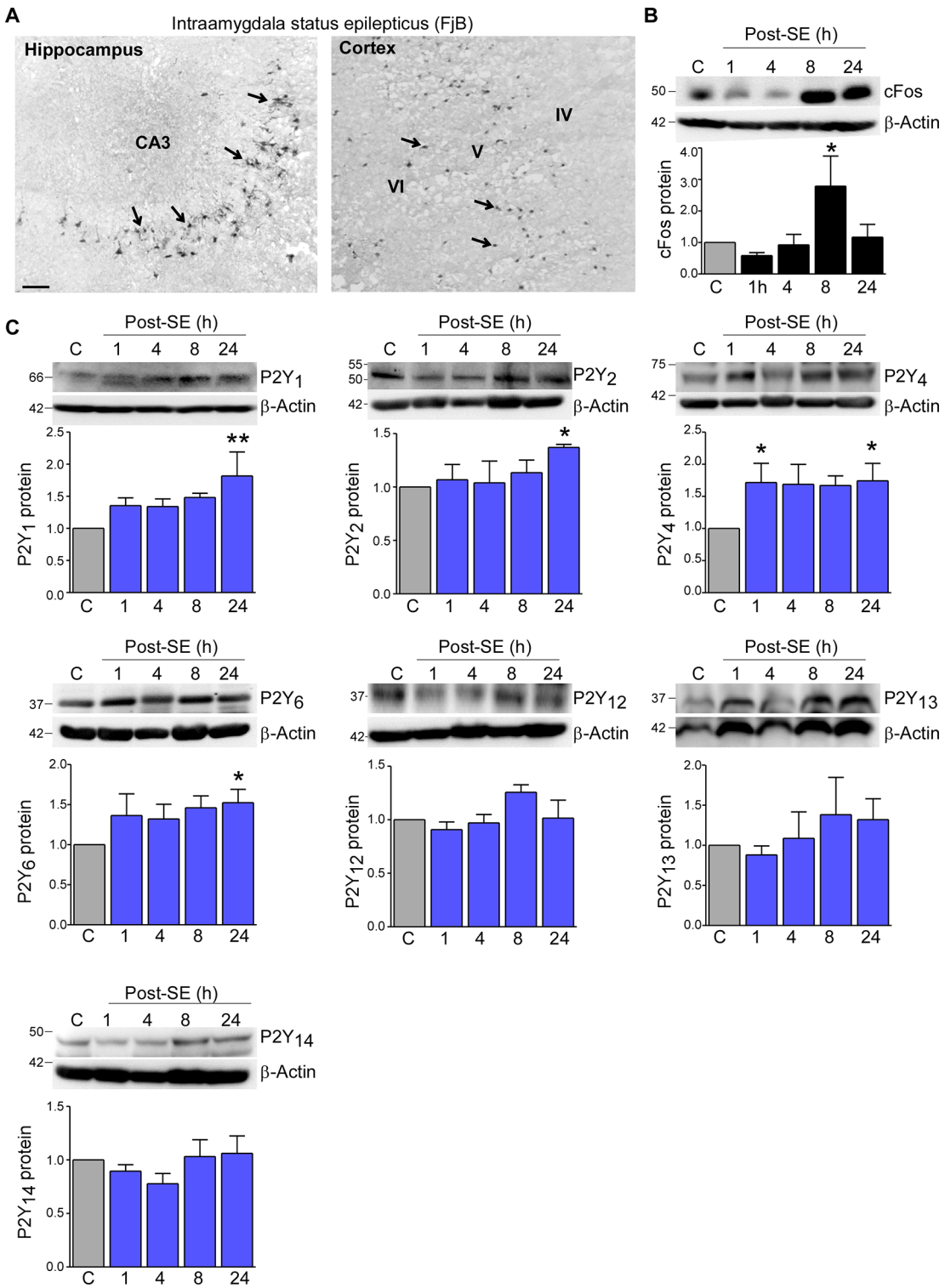

FIGURE 1 | Expression profiling of the P2Y receptor family in the cortex following status epilepticus. **(A)** Photomicrograph (20× lens) showing neuronal damage 24 h following intraamygdala KA-induced status epilepticus in the ipsilateral hippocampus and cortex. Scale bar = 100 μm. **(B)** Representative Western blot (n = 1 per lane) and corresponding graph showing increased c-Fos expression in the ipsilateral cortex post-status epilepticus (n = 4 per group). **(C)** Representative Western blots (n = 1 per lane) and corresponding graphs showing the expression of the different P2Y receptor family members $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ in cortical tissue following status epilepticus. Of note, while $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ are significantly increased post-status epilepticus, no changes could be observed for the remaining P2Y receptors (n = 6 per group). $^*p < 0.05$, $^{**}p < 0.01$.

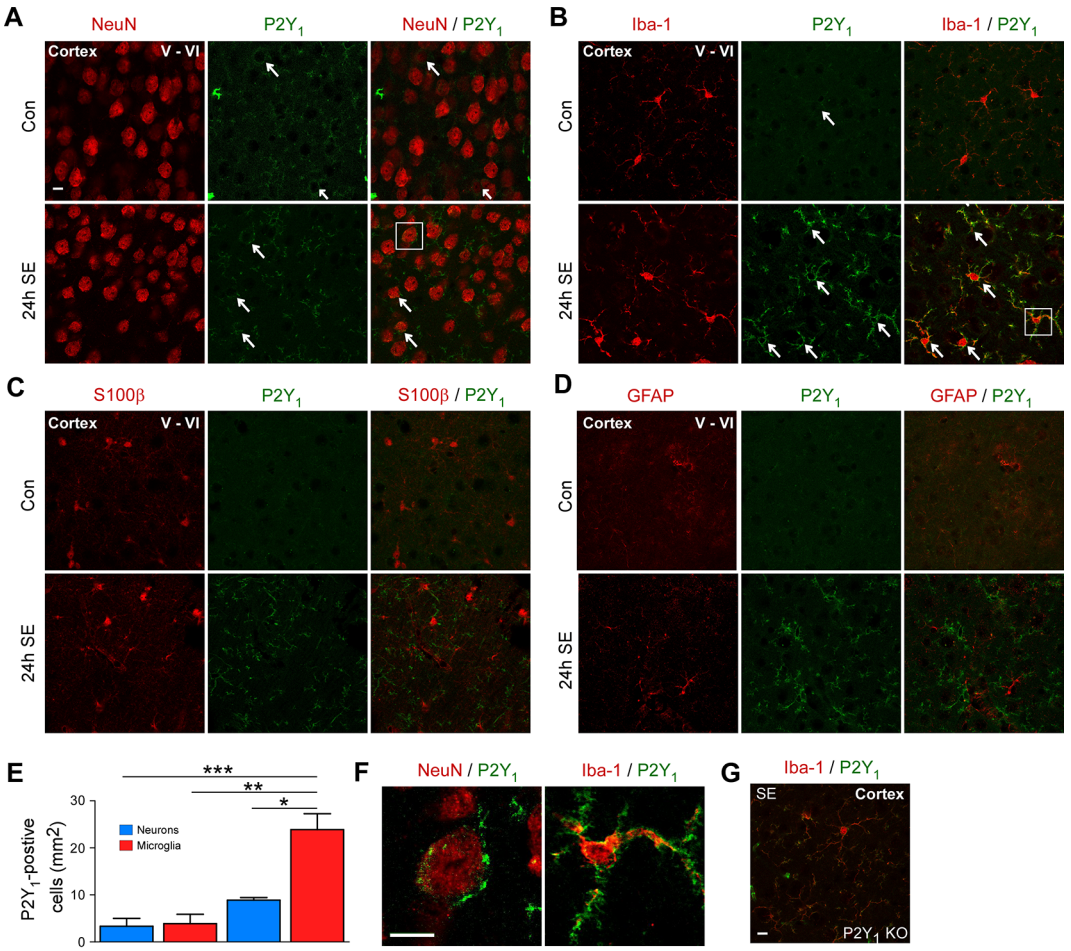

FIGURE 2 | Cell-specific expression of $P2Y_1$ post-status epilepticus. **(A)** Photomicrographs (40× lens) showing co-localization of $P2Y_1$ (green) with neuronal marker NeuN in cortical tissue (layer V–VI) in control injected-vehicle mice and following status epilepticus (SE) (white arrows). Scale bar = 10 μm **(B)** Co-localization of $P2Y_1$ (green) with microglia marker Iba1 (red) 24 h following status epilepticus (SE) in the cortex (white arrows). Scale bar = 10 μm. **(C, D)** No co-localization of $P2Y_1$ (green) with the astrocyte markers GFAP and S100β (red). Scale bar = 10 μm. **(E)** Graph showing strong increase in $P2Y_1$-positive microglia 24 h following status epilepticus (n = 3 per group). **(F)** Enlarged images, outlined by white box, showing co-localization of $P2Y_1$ (green) on neurons (red) and microglia (red) 24 h post-status epilepticus (SE). Scale bar = 10 μm. **(G)** Specificity of $P2Y_1$-detecting antibody was confirmed using tissue from $P2Y_1$ knockout mice (KO) 8 h post-status epilepticus. Scale bar = 10 μm. Images are representative image from three animals per experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

brain tissue was stained with the neurodegeneration marker FjB and cell death quantified in the cortex as before (Jimenez-Pacheco et al., 2013). While no significant effect on cortical neurodegeneration could be observed in mice treated with the $P2Y_1$ agonist MRS23 (**Figure 4A**), treatment with the $P2Y_1$ antagonist MRS25 reduced significantly neurodegeneration in the cortex (**Figure 4A**). Conversely, mice pre-treated with minocycline and then treated with the $P2Y_1$ antagonist MRS25 during status epilepticus showed no difference in neurodegeneration in the cortex when compared to minocycline pre-treated vehicle-injected mice subjected to status epilepticus (**Figure 4B**).

In conclusion, our result suggests that $P2Y_1$ antagonism not only reduces seizure severity during status epilepticus, but also protects the brain from damage including cortical tissue.

DISCUSSION

In the present study, by using a unilateral mouse model of status epilepticus, we report an overall increase in the expression of the P2Y receptor family in the cortex following status epilepticus and that $P2Y_1$ antagonism reduces harmful HFHA polyspiking during status epilepticus and protects the cortex from seizure-induced neurodegeneration. Finally, we show that the observed neuroprotective effect provided by $P2Y_1$ antagonism is mediated, at least in part, *via* reducing inflammation in the brain. Therefore, our study extends previous data demonstrating a detrimental role of $P2Y_1$ activation during status epilepticus and further suggests that targeting of $P2Y_1$ may represent a novel therapeutic avenue to treat patients with drug-refractory status epilepticus.

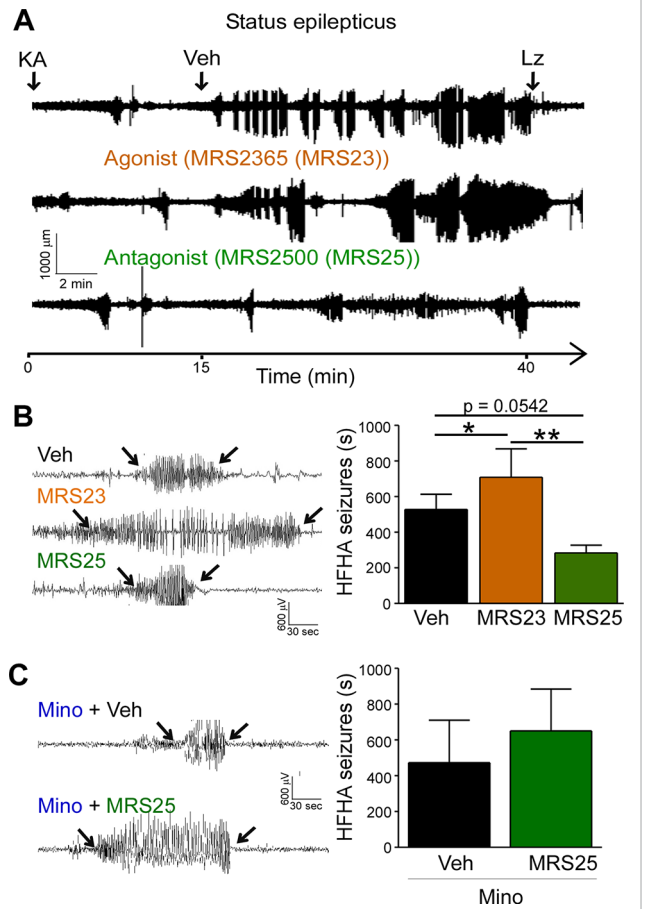

FIGURE 3 | $P2Y_1$ antagonism decreases high frequency high amplitude spiking during status epilepticus. **(A)** Representative EEG traces recorded from the cortex from the time-point of intraamygdala kainic acid (KA) injection until 60 min post-lorazepam (Lz) of mice treated with vehicle (Veh), $P2Y_1$ agonist MRS2365 (MRS23), and $P2Y_1$ antagonist MRS2500 (MRS25). Treatment with $P2Y_1$-targeting drugs was administered 15 min post-intraamygdala KA injection *via* i.c.v. Lorazepam (Lz) was administered 40 min following KA injection *via* i.p. **(B)** Representative EEG traces showing examples of high frequency and high amplitude (HFHA) spiking taken during the 30 min recording period following drug treatment (see arrows). Mice treated with the $P2Y_1$ agonist MRS23 showed an increase in the duration of HFHA spiking while mice treated with the $P2Y_1$ antagonists MRS25 showed a decrease in the duration of HFHA spiking during status epilepticus (n = 7 Veh, 9 MRS23, and 8 MRS25). **(C)** Representative EEG traces and graph showing slightly increased duration of HFHA spiking during status epilepticus in mice treated with minocycline and $P2Y_1$ antagonists MRS25 (n = 4 per group). $^*p < 0.05$, $^{**}p < 0.01$.

While there was little interest in P2Y receptors as potential drug-targets to treat status epilepticus or epilepsy, this has changed significantly over the past years with emerging evidence suggesting a causal role of this receptor family not only during seizure generation, but also in the development of epilepsy (Alves et al., 2018). Recent data published by us has demonstrated a distinct expression profile of the P2Y receptor family in the hippocampus following status epilepticus in both animal models and temporal lobe epilepsy patients (Alves et al., 2017). We have now extended these data analyzing the cortex, a structure in which status epilepticus also induces neuronal death (Curia et al., 2008; Mouri et al., 2008). Here we show that status epilepticus leads to an up-regulation of $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ in the cortex, with the $P2Y_1$ and $P2Y_4$ receptors showing the strongest increase. These results are in line with a former study where we have shown an upregulation of the Gq-binding P2Y receptors in the hippocampus post-status epilepticus (Alves et al., 2017). The observed status epilepticus-induced decrease in $P2Y_{12}$ expression in the hippocampus could, however, not be replicated in the cortex. Moreover, P2Y expression changes occurred at later time-points following status epilepticus in the cortex when compared to the hippocampus with the only exception being $P2Y_4$ which was increased shortly following status epilepticus in both brain structures. We do not know what the reason for these discrepancies between different brain areas are; however, different brain structures are differently affected by seizures during status epilepticus with seizures first occurring in the hippocampus when compared to the cortex (Engel et al., 2017). Differences in cell populations between cortex and hippocampus may further contribute to differences observed in the expression profile between both brain structures.

Indirect evidence suggesting a functional role for $P2Y_1$ and $P2Y_4$, P2Y receptors undergoing the strongest increase in their expression following status epilepticus, stems from a study showing that treatment with ADP, main endogenous $P2Y_1$ agonist, increases seizure severity during status epilepticus and treatment with UTP, main endogenous agonists of $P2Y_4$, decrease seizure severity (Alves et al., 2017). In line with this, $P2Y_1$-targeting has been repeatedly shown to protect against status epilepticus using different experimental models of seizures and epilepsy (Alvarez-Ferradas et al., 2015; Nikolic et al., 2018; Simoes et al., 2018; Alves et al., 2019). However, in contrast to UTP-binding receptors being anticonvulsive, a more recent study using a rat model of KA-induced acute seizures has shown that blocking $P2Y_4$ reduces seizure severity during status epilepticus (Zhang et al., 2019). Future studies using different mouse models of status epilepticus and different treatment regimens (pre-treatment vs. post-treatment) will have to clarify whether these observed effects of targeting $P2Y_4$ are model- and/or treatment-specific. Nonetheless, the increased expression of both receptor subtypes following status epilepticus and during epilepsy suggests drugs targeting these receptors as possible therapeutic approaches for both drug-refractory status epilepticus and epilepsy.

Previously we have shown that in the mouse hippocampus $P2Y_1$ is expressed in neurons under normal physiological condition and, following status epilepticus, is also detected on microglia (Alves et al., 2019). In the cortex, $P2Y_1$ receptor expression was mainly detected on cortical neurons during physiological control conditions and post-status epilepticus, although at somewhat lower levels when compared to the

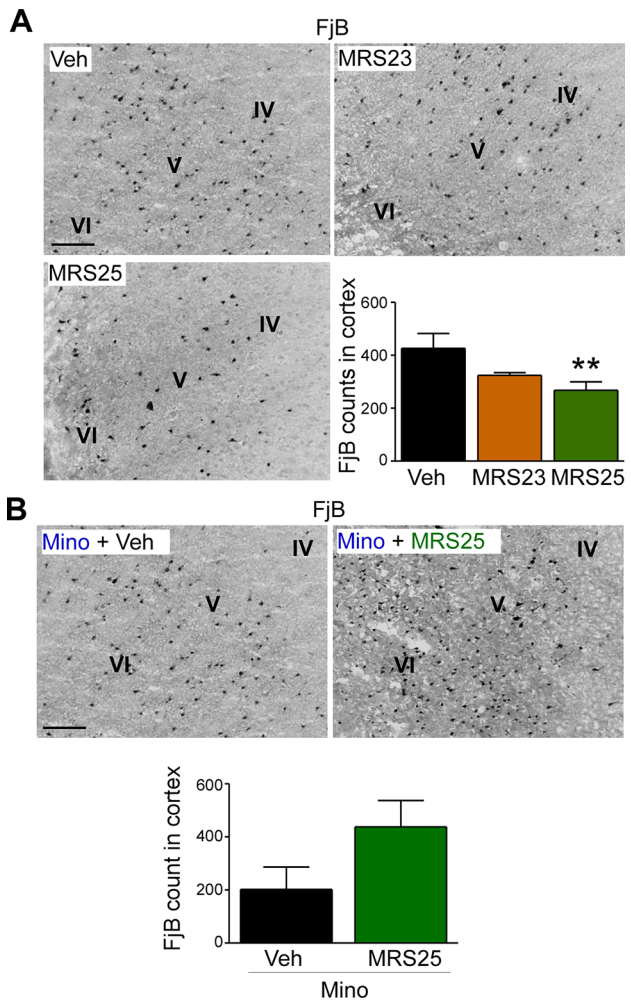

FIGURE 4 | Decreased status epilepticus-induced cortical damage *via* $P2Y_1$ antagonism. **(A)** Representative images (20× lens) and corresponding graph showing a decrease in neuronal damage in the cortex 24 h post-status epilepticus in mice treated with the $P2Y_1$ antagonist MRS25 (n = 7 Veh, 9 MRS23, and 8 MRS25). **(B)** Representative images (20x lens) and corresponding graph showing slightly more Fluoro-Jade B (FjB)-positive cells in ipsilateral cortex 24 h post-status epilepticus in mice treated with both minocycline and the P2Y1 antagonist MRS25 (n = 4/group). $^{**}p < 0.01$.

hippocampus (Alves et al., 2019). In cortical microglia, $P2Y_1$ expression was, however, as observed before in the hippocampus, almost undetectable during control conditions and strongly increased 24 h following status epilepticus (De Simone et al., 2010; Alves et al., 2019). Although $P2Y_1$ has been described to be expressed on astrocytes under different pathological conditions, such as oxidative stress (Shinozaki et al., 2006; Fujita et al., 2009), ischemia (Zheng et al., 2013), and in patients with cortical dysplasia (Sukigara et al., 2014), no expression of $P2Y_1$ was detected on astrocytes in the cortex in our status epilepticus mouse model. This is in agreement with our previous study showing absence of $P2Y_1$ on astrocytes in the hippocampus post-status epilepticus (Alves et al., 2019). We included in our study a staining performed in the $P2Y_1$ KO mouse as a negative control, demonstrating the specificity of the $P2Y_1$ antibody. $P2Y_1$ expression on cortical astrocytes may also be below the detection range of our antibody-based detection methods and electrophysiological techniques may be required. However, regardless whether $P2Y_1$ is present on astrocytes or not, our results show that $P2Y_1$ is also in the cortex strongly upregulated on microglia, suggesting $P2Y_1$-driven microglia activation during status epilepticus not being restricted to the hippocampus.

Here, we also report that while treatment with $P2Y_1$ agonists increases HFHA polyspiking during status epilepticus, $P2Y_1$ antagonism reduces HFHA polyspiking. This is in line with previous findings showing a reduction in total severity of seizures during status epilepticus *via* $P2Y_1$ antagonism (Alves et al., 2019). Suggesting this being neuroprotective, studies in the intraamygdala KA mouse model of status epilepticus have shown HFHA spiking to correlate with brain injury (Araki et al., 2002). In line with HFHA spiking causing neurodegeneration, mice treated with the $P2Y_1$ antagonist MRS25 also showed less cell death in the cortex. However, despite the increase in HFHA polyspiking during status epilepticus caused by the $P2Y_1$ agonist MRS23, this did not translate into more cell death in the cortex. The reason for this remains elusive; $P2Y_1$ may have, however, effects independent on increasing hyperexcitability which impact on cell survival. In line with $P2Y_1$ being anti-apoptotic, we have recently shown that $P2Y_1$ overexpression protected against KA-induced neuronal death *in vitro* (Alves et al., 2019).

Finally, demonstrating $P2Y_1$ contributing to seizure pathology at least in part *via* driving inflammation, anticonvulsive and neuroprotective effects conferred by $P2Y_1$ antagonism were lost when mice were pre-treated with minocycline, which is in good agreement with our previous results examining the effects of $P2Y_1$ signaling on the hippocampus (Alves et al., 2019). It has to be noted that minocycline treatment reduced cortical neurodegeneration following status epilepticus. It is, however, unlikely that this reduction in cell death had an impact on our results as both groups were pre-treated with minocycline. The cell-specific contribution of $P2Y_1$ to the observed effects remains, however, to be elucidated. It is tempting to speculate that effects provided by $P2Y_1$ antagonism are due to $P2Y_1$ driving microglia activation. Indeed, our results show a dramatic increase in $P2Y_1$ immunoreactivity on microglia post-status epilepticus and the broad-spectrum anti-inflammatory drug minocycline has been shown to act predominately on this glial cell type (Abraham et al., 2012; Alves et al., 2019). Importantly, a role for $P2Y_1$ on microglia activation has been repeatedly demonstrated previously (Davalos et al., 2005; Farber and Kettenmann, 2006; De Simone et al., 2010) with studies showing $P2Y_1$-mediated signaling on microglia to affect neurodegeneration during ischemia and traumatic brain injury (Shinozaki et al., 2017; Fukumoto et al., 2018). Moreover, microglia are the first cells to respond during brain inflammation

and microglia also respond rapidly to acute neuronal hyperactivity during seizures *via* NMDA-type glutamate receptors (Davalos et al., 2005; Eyo et al., 2014). We cannot rule out, however, a contribution of other cell types such as neurons or astrocytes. To fully prove the cell-specific contribution to $P2Y_1$-mediated effects, this would require the use of cell-specific $P2Y_1$-deficient mice. Nevertheless, our results strongly suggest that the anticonvulsive and neuroprotective effects mediated *via* $P2Y_1$ signaling are due to $P2Y_1$ driving inflammatory processes.

In conclusion, our study extends previous data confirming anticonvulsive and neuroprotective properties of $P2Y_1$ antagonism during status epilepticus, further suggesting $P2Y_1$-based treatment as possible new therapy for drug-resistant status epilepticus.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

All animal studies were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland.

AUTHOR CONTRIBUTIONS

MA carried out Western blotting and EEG analysis and wrote parts of the manuscript. JS performed immunohistochemistry and wrote parts of the manuscript. TE supervised study, carried out *in vivo* work, and wrote the manuscript.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Signaling of the Purinergic System in the Joint

Carmen Corciulo [1,2] *and Bruce N. Cronstein* [1,3]*

[1] Division of Translational Medicine, Department of Medicine, NYU School of Medicine, New York, NY, United States, [2] Krefting Research Centre—Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden, [3] Division of Rheumatology, Department of Medicine, NYU School of Medicine, New York, NY, United States

The joint is a complex anatomical structure consisting of different tissues, each with a particular feature, playing together to give mobility and stability at the body. All the joints have a similar composition including cartilage for reducing the friction of the movement and protecting the underlying bone, a synovial membrane that produces synovial fluid to lubricate the joint, ligaments to limit joint movement, and tendons for the interaction with muscles. Direct or indirect damage of one or more of the tissues forming the joint is the foundation of different pathological conditions. Many molecular mechanisms are involved in maintaining the joint homeostasis as well as in triggering disease development. The molecular pathway activated by the purinergic system is one of them.The purinergic signaling defines a group of receptors and intermembrane channels activated by adenosine, adenosine diphosphate, adenosine 5'-triphosphate, uridine triphosphate, and uridine diphosphate. It has been largely described as a modulator of many physiological and pathological conditions including rheumatic diseases. Here we will give an overview of the purinergic system in the joint describing its expression and function in the synovium, cartilage, ligament, tendon, and bone with a therapeutic perspective.

Keywords: adenosine, cartilage, bone, synovium, tendon, purine, adenosine triphosphate, adenosine diphosphate

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***Correspondence:**
Bruce N. Cronstein
bruce.cronstein@nyumc.org

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ANATOMY AND FUNCTION OF THE JOINT

The joint, the structure around the potential space between bones, is a complex anatomical structure consisting of several different types of tissues that together, permit mobility and stability at the body (**Figure 1**). Joints located in different anatomical areas have their own structure which evolved to counteract the different intensities of mechanical loading and the need for fine movements.

A first joint classification is based on the features of the link between two bones: in the fibrous joint, there is no cavity between adjacent bones connected by fibrous tissue; in the diarthrotic joint movement can take place freely in various planes; the amphiarthrosis joint is slightly movable and ligaments connect the bones. The diarthrosis joints are surrounded by an articular capsule composed of connective tissue filled with synovial fluid allowing free movement and giving stability protecting from dislocation. Bones provide stability and support the muscle. The skeleton is kept in appropriate alignment by ligaments and connected to the muscle by tendons (Anderson and Bordoni, 2019).

Many pathological conditions directly or indirectly affect the joint such as osteoarthritis and rheumatoid arthritis (RA) and they may be due to aging, mechanical stress, or injury as in the case of ligament rupture, or to general inflammatory status as in rheumatoid arthritis (Lieberthal et al., 2015; Tracy et al., 2017).

Appropriate interactions between these different tissues are carried out at the cellular and molecular level by numerous molecules and molecular pathways activated and the purinergic system is one of them (Rumney et al., 2012; Cronstein and Sitkovsky, 2017).

In this review, we will describe the expression and function of the purinergic system in the joint.

PURINERGIC SYSTEM

The purinergic signaling is activated by the extracellular binding of adenosine, adenosine diphosphate (ADP), adenosine triphosphate (ATP), uridine triphosphate (UTP), and uridine diphosphate (UDP) to transmembrane receptors and ions channels. This system was characterized in the early 1990s with the description of four G-protein coupled (GPCR) adenosine receptors (P1), seven P2X ion channels activated by ATP, and eight P2Y G-protein coupled receptors, binding sites for ATP, ADP, UTP, and UDP (Menzies et al., 2017; Burnstock, 2017).

In the resting state the amount of ATP in the cytosol is between 1 mM and 5 mM whereas it is much lower in the extracellular space ranging in the nanomolar concentration (10–100 nM) (Bours et al., 2006). The extracellular nucleotides are released massively from the cytosol as a "danger" signal and their concentration increases during apoptosis, mechanical stress, e.g. stretch and shear stress, in hypoxic conditions and during infection. ATP can be released into the extracellular space by vesicular transport or through channels localized on the cell membrane, like Pannexin and Connexin channels, or it can reach the extracellular space after cell lysis (Di Virgilio et al., 2009). ATP and ADP bind and activate their own receptors: P2Y receptors, including eight subtypes of metabotropic receptors coupled to G-proteins, seven P2X hetero or homotrimers forming ion channels permeable to Na+, K+, and Ca2+ upon ATP binding (Abbracchio et al., 2006; Kaczmarek-Hajek et al., 2012; Sluyter, 2015). Prolonged activation of P2X receptors can lead to conformational changes in the structure of the trimers leading to higher ion permeability or to channel desensitization, an effect depending on the receptor subtype activated (Khakh et al., 1999; Saul et al., 2013). The functionality of P2Y receptors is instead regulated by the recruitment of β-arrestin (subtypes 1–4) which promotes inhibition of the interaction receptor G-protein (desensitization), receptor internalization or activates the signaling cascade. Which subtype of β-arrestin is recruited and the intensity of the interaction depends on the receptor-activated and on the ligand involved, ATP or UTP (Erb and Weisman, 2012).

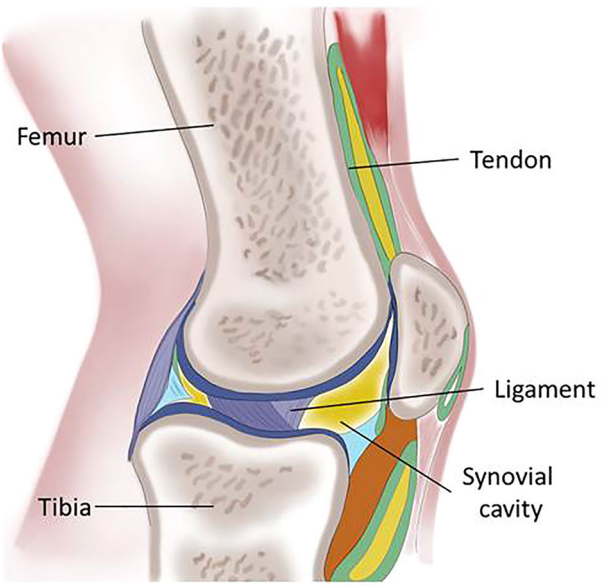

FIGURE 1 | Schematic representation of the knee describing the main tissues of the articular joint: femur and tibia, the skeletal elements are maintained in position by the ligaments and connected to the muscles by the tendons; the synovial cavity is lined by the synovial membrane.

ATP is also the source of adenosine. Adenosine is released into the extracellular space by most cells and tissues. The basal level of adenosine in extracellular fluids is roughly 100 nM and its concentration increases during periods of cell stress rising into the low micromolar range. This increase in concentration is the result of more adenosine released through the equilibrative nucleoside transporter (ENT) or, more importantly, release of adenine nucleotides which are hydrolyzed by a series of cell surface and soluble enzymes, including ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1; from ATP to ADP and AMP) and ecto-5`-nucleotidase, also known as CD73, that converts AMP into adenosine. Finally, adenosine in the extracellular space can be metabolized to inosine by adenosine deaminase (ADA) or taken up by cells and rephosphorylated by adenosine kinase (Hasko et al., 2018) (**Figure 2**).

There are four subtypes of adenosine receptors named A1, A2A, A2B, and A3 which are all members of the large family of G protein-coupled receptors (GPCRs). A1 and A3 receptors are coupled to Gi signal transduction proteins which inhibit adenylate cyclase; A2A and A2B receptors promote cAMP synthesis by coupling to Gs protein; A2BR is also coupled to Gq protein (Ryzhov et al., 2006). Adenosine binds A1 and A2A receptors with an affinity of 10–30 nM; the affinity for A3 receptor is roughly 1 uM and even higher for the A2BR (Fredholm et al., 2011). The downstream signaling receptor function is regulated by β-arrestin that, preventing interaction of the receptor with the G proteins, switches off the signaling and mediates internalization of the receptor (Verzijl and Ijzerman, 2011).

The purinergic system aroused interest in the rheumatology and orthopedic field for its direct effect on the joint and indirect

effect on articular tissues mediated by modulation of the immune response.

In the next paragraphs, we will describe the direct effect of the purinergic system activation on the different joint tissues.

THE PURINERGIC SYSTEM IN THE JOINT

Cartilage

P2 Receptors

P2 receptors were first identified in human chondrocytes in 1991 as mediators of prostaglandin E2 release (Caswell et al., 1991) and more recently there is a deep focus in the field on the role of ATP in mechanotransduction and cartilage homeostasis (Millward-Sadler et al., 2004; Knight et al., 2009; Garcia and Knight, 2010).

The activation of P2 receptors following mechanically induced ATP release or addition of exogenous ATP has an anabolic effect, with up-regulation in proteoglycan, collagen synthesis, and cell proliferation (Brown et al., 1997; Croucher et al., 2000; Picher et al., 2003; Millward-Sadler et al., 2004; Chowdhury and Knight, 2006). In contrast, it has also been reported that ATP stimulates proteoglycan breakdown and glycosaminoglycan release from bovine nasal cartilage explants and thus, may have a role in diseases that primarily involve the destruction of non-articular cartilage (Leong et al., 1990; Brown et al., 1997). Interestingly the opposite effect was measured when a similar experiment was performed on articular cartilage confirming that the heterogeneous results depend on the tissues, articular vs. non-articular cartilage, involved (Brown et al., 1997). In fact, bovine primary articular chondrocytes culture supplemented with ATP increases neocartilage collagen by 110% (Garcia and Knight, 2010).

In pathological conditions, high levels of ATP have been associated with cartilage degradation and pathological calcification. Mutation in the progressive ankyloses gene, ANK, leads to calcium pyrophosphate dihydrate crystal deposition (CPPD) in the joint and consequent cartilage degradation. Overexpression of ANK correlates with an increase of extracellular ATP (Rosenthal et al., 2013). Because of this preliminary evidence, Probenecid, a drug in common use for the treatment of gout and which blocks ATP release by Pannexin1 and ANK channels, is currently in clinical testing in CPPD patients (https://clinicaltrials.gov/ct2/show/NCT02243631) (Silverman et al., 2008).

The articular cartilage is subject and responsive to mechanical stimuli. Mechanical force on the extracellular matrix of the cartilage is transferred to the chondrocytes through integrins such as integrin B3, that transfer the stimuli at molecular level activating homeostatic mechanisms and compensating for the mechanical stress (Kudirka et al., 2007). Applying a 1 Hz mechanical force to a bovine chondrocyte culture in agarose produces a sevenfold increase of ATP release by the opening of the hemichannels (Garcia and Knight, 2010). Moreover, fluid flow shear stress increases secretion of ATP through connexin hemichannels promoting lubricin production (Graff et al., 2000; Millward-Sadler et al., 2004; Bao et al., 2004; Gomes et al., 2005; Ogawa et al., 2014). According to these studies it has been demonstrated that bovine and human chondrocytes express connexin 43 (Knight et al., 2009) and blockade of connexin hemichannels in chondrocyte culture reduces extracellular ATP and collagen II production (Schrobback et al., 2015). Moreover experiments performed on primary chondrocytes subjected to cyclic strain and mice subjected to treadmill running confirm that extracellular ATP is an important mediator of mechanotransduction which downregulates the expression of metalloproteinases MMP-1 and MMP-13 (He et al., 2016). Conversely, another research group demonstrated that prolonged cyclic axial compression of chondrocytes suppressed the extracellular ATP level and contributed to the destabilization of cartilage (Coleman et al., 2016). Human chondrocytes stimulated with a mechanical force of 0.33 Hz hyperpolarize through a mechanism mediated by ATP release and P2Y receptors activation. Interestingly, no change in membrane polarization was measured in OA chondrocytes (Millward-Sadler et al., 2004). Moreover we have demonstrated that incubation of murine chondrocytes with IL-1β decreases ATP release (Corciulo et al., 2017). All these results document the

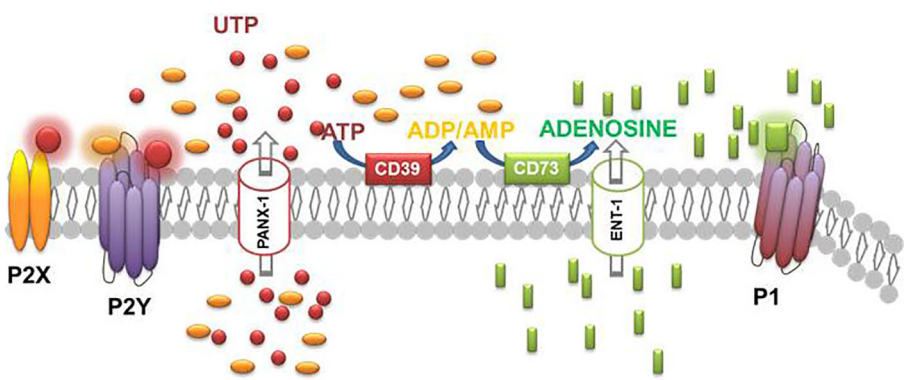

FIGURE 2 | Schematic diagram illustrating the effectors, enzymes, channels, and receptors playing a role in the purinergic system.

impairment of the ATP signaling during OA development and inflammation of the joint suggesting a constitutive activation of P2 receptors in chondrocytes is associated with cartilage protection and maintenance of the homeostasis of the extracellular matrix.

Because different studies have yielded different results it is not clear whether ATP released in physiological conditions into the extracellular space protects or mediates cartilage damage during mechanical stress. These contradictory findings suggest that the response to ATP may either be dose-dependent or that the heterogeneous response depends on the health status of the joint, on which cartilage (articular or non-articular) is involved and what kind of mechanical forces are applied to the tissues (**Figure 3**).

P1 Receptors

The presence of P1 receptors on chondrocytes was demonstrated in 1999 by Koolpe and colleagues in human articular chondrocytes (Koolpe et al., 1999) and the homeostatic role of adenosine in cartilage and chondrocytes has been described by our and other laboratories. All adenosine receptors are expressed in bovine, mouse, and human articular chondrocytes (Varani et al., 2008; Varani et al., 2008; Vincenzi et al., 2013). Adenosine is responsible for reduced NO production in equine cartilage explants incubated with LPS, an effect mediated by ligation of A2AR and confirmed by incubation of chondrocytes with adenosine deaminase or an A2AR antagonist. Moreover it has been documented that A2AR agonists increase cAMP in equine chondrocytes and reduce the Il-1β mediated TNF-α, IL-6, MMP-13, and NO production (Tesch et al., 2002; Campo et al., 2012). The depletion of adenosine by adenosine deaminase also increases the production of metalloproteinases, PGE2 and NO, shedding light on the homeostatic role of adenosine in healthy cartilage and suggesting a constitutive activation of its receptors (Tesch et al., 2002; Benton et al., 2002; Tesch et al., 2002; Mistry et al., 2006). In our laboratory we demonstrated that adenosine is an important homeostatic regulator of chondrocytes during inflammation. We showed that in chondrocytes and rat cartilage explants transcripts of CD73 and channels for adenosine and ATP release are down-regulated after IL-1β incubation as well as ATP and adenosine concentrations in the extracellular space. The replacement of adenosine in the intraarticular space has shown delayed osteoarthritis progression in a rat model of post-traumatic osteoarthritis through a molecular mechanism involving A2A adenosine receptor activation (Corciulo et al., 2017). We also demonstrated that mice lacking A2A adenosine receptors develop spontaneous bone and cartilage features of OA associated with reduced motor activity and Shkhyan and colleagues demonstrated that ablation of A3 receptors results in OA development in aged mice (Corciulo et al., 2017; Shkhyan et al., 2018) (**Figure 3**).

Synovial Tissue

The synovial tissue is a two cell layers thick membrane containing mainly two kinds of cell populations: synovial tissue macrophages (type A cells) and fibroblast-like synoviocytes (type B cells) (Falconer et al., 2018). Infiltration of lymphocytes, mild in OA and extensive in RA, leads to tissue hyperplasia contributing to inflammation, neovascularization, cartilage degradation, and pain sensitization (Mor et al., 2005; Asif Amin et al., 2017; Belluzzi et al., 2019). Activated synovial fibroblasts are involved in cartilage damage, particularly in RA subjects, *via* production of IL-6, IL-8, and TNF-α.

P2 Receptors

ATP release into the synovial fluid is triggered by the hypoxic environment in the synovium and hypotonic nature of the synovial fluid typical of RA. When synoviocytes *in vitro* were challenged with a hypotonic shock they activated a mechanism of calcium-mediated ATP release leading to reduced cell viability and synovium membrane hyperplasia (Hu et al., 2017). A study in 28 OA patients demonstrated that ATP concentration in the synovial fluid positively correlates with pain experienced by the patients. Moreover ATP concentration decreased after hyaluronic acid treatment (Kumahashi et al., 2011). Similar results were also demonstrated in OA dogs (Torres et al., 2016).

Synoviocytes isolated from RA patients express P2X1, P2X2, P2X3, P2X4, P2X5, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14 receptors whereas in synovial cells from OA patients two membrane ion channels, P2X(1) and P2X(3) receptors, were present (Caporali et al., 2008; Varani et al., 2010). In the synovial membrane ATP also induces the production of brain-derived neurotrophic factor, a neuromodulator involved in nociceptive hypersensitivity, by activating P2X4 receptor (Klein et al., 2012). The same receptor promotes IL-1β release from SF

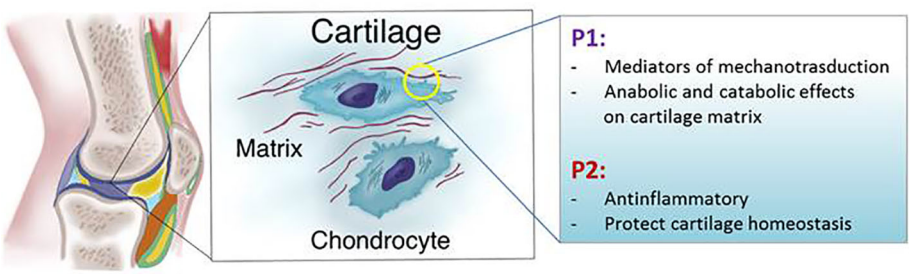

FIGURE 3 | Schematic representation of P1 and P2 receptors functions in articular cartilage.

isolated from OA patients (Fan et al., 2014). On the contrary, P2X1 receptors showed an anti-inflammatory effect reducing NF-kB activation and TNF-α release whilst P2X3 receptors mediated the opposite response (Varani et al., 2010).

P2X7 receptor expression was detected in inflamed synovial tissue in a rat arthritis model and inhibition of this receptor reduced articular inflammation and progressive bone destruction in a rat model of collagen-induced arthritis, an effect due to the attenuation of the leukocytes inflammatory response (Cronstein et al., 1993; McInnes et al., 2014). Despite the positive results in the pre-clinical study, antagonists for P2X7 receptor, CE-224,535 and AZD9056, did not show any efficacy when administrated orally to patients with rheumatoid arthritis (Montesinos et al., 2000; Montesinos et al., 2003) (**Figure 4**).

P1 Receptors

mRNA of all adenosine receptors are expressed in human SF where they exert an anti-inflammatory function (**Figure 4**). In particular adenosine inhibits the pro-inflammatory pathway activated by NF-kB through binding to A2AR and A3R (Varani et al., 2010) whereas it has been shown that the anti-inflammatory effect of the electromagnetic field on synovial fibroblasts is mediated by A1R and A2AR upregulation (De Mattei et al., 2009).

Another evidence of the anti-inflammatory role of adenosine comes from the measurement of ADA levels in the synovial fluid, higher in the RA patients compared to the OA patients, and correlating with the disease severity (Sari et al., 2003). Nevertheless treatment of RA synoviocytes with 2-chloroadenosine (2-CADO), an adenosine analog resistant to adenosine deaminase, induces cell apoptosis independent from adenosine receptors activation. Interestingly adenosine does not have the same of 2-CADO effect on cell apoptosis (Koshiba et al., 2002) pointing out that regulation of adenosine amount in the extracellular space is fundamental to avoid cell toxicity. It is a matter of fact that patients with adenosine deaminase 2 deficiency present vascular symptoms and rheumatic inflammatory features (Hashem et al., 2017).

Fibroblasts like synoviocytes isolated from RA patients express all adenosine receptors. In RA A3R is expressed significantly more than the other receptors while A2AR is less expressed (Stamp et al., 2012). Targeting A3R to promoting activation of anti-inflammatory mechanisms has been proposed since the A3R agonist, CF101, showed efficacy in a pre-clinical RA model and in RA patients reduces their symptoms and signs (Silverman et al., 2008; Bar-Yehuda et al., 2009).

Adenosine exerts an indirect role of joint protection by modulating the pro-inflammatory NF-kB molecular signaling in immune cells. Increased expression of A2AR and A3R adenosine receptors has been measured in white cells of RA and in ankylosing spondylitis patients, an effect that decreased after anti TNF-alpha treatment, consistent with the known effect of TNF-alpha on the expression of adenosine A2AR *via* NF-kB activation (Varani et al., 2009; Varani et al., 2011; Ravani et al., 2017). Moreover, the effect of low dose, weekly methotrexate (MTX), the gold standard treatment for RA, is also mediated by increased released of adenosine and activation of A2AR and A3R. It is well established that MTX induces the release of adenosine and reduces inflammation in an adenosine-dependent manner (Cronstein et al., 1993). Initially, it was demonstrated that only non-selective antagonists for adenosine receptors were able to inhibit MTX anti-inflammatory effect consistent with the involvement of multiple receptors (Montesinos et al., 2000). Further studies suggested that low doses of MTX, comparable to the doses used in RA patients, were able to activate A2AR and A3R (in the air pouch model) and only A2AR in a model of peritoneal inflammation (Montesinos et al., 2003; Montesinos et al., 2006).

Bone

Bone is a dynamic tissue subjected to continuous remodeling, adjusting to environmental changes and responding to inflammation, hormonal alterations, and mechanical injury in pathological conditions (Rowe and Sharma, 2019). Osteoblasts, osteoclasts, and osteocytes are the principal cell types that interact and cooperate at the molecular level to orchestrate bone remodeling (Olsen et al., 2000). Osteoclasts are responsible for bone resorption and formation of resorption lacuna. They are differentiated from hematopoietic cells by a molecular mechanism controlled mainly by two cytokines, macrophage colony-stimulating factor M-CSF and the ligand for the receptor activator of nuclear factor kB (RANKL) (Kim and Kim, 2016). Osteoclasts polarize when they are in contact with a mineralized matrix forming specific domains including

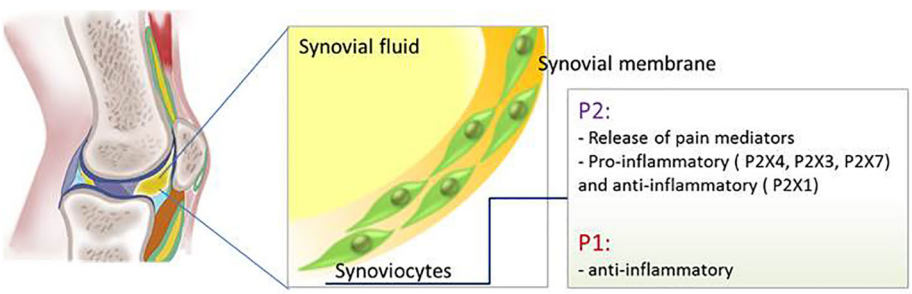

FIGURE 4 | Schematic representation of P1 and P2 receptors functions in the synovial membrane.

the sealing zone, the ruffled border, and the functional secretory domain, all important for creating a compartment for bone reabsorption (Martin and Sims, 2005; Crockett et al., 2011). Osteoclasts synthesize MMP-2, 4, 6, and 7 important for osteoblasts recruitment and activation (Garimella et al., 2008). Osteoblasts, instead, differentiate from mesenchymal precursors in the bone marrow (Ponte et al., 2007; Uccelli et al., 2008; Garg et al., 2017). During bone formation osteoblast precursors interact with the bone surface to generate a calcified matrix (Marie et al., 2014). Osteocytes are embedded in the bone matrix and are connected to each other by long dendritic cell extensions through a network made by canaliculi through which they sense changes in mechanical loading and regulate the function of osteoblasts and osteoclasts (Hemmatian et al., 2017).

The regulation of the bone homeostasis is heavily dependent on the purinergic system (**Figure 5**).

P2 Receptors

ATP release and P2 receptor signaling are key modulators of skeletal development and homeostasis. Pannexin and connexin channels, P2X and P2Y receptors were detected in osteoblasts, osteoclasts, and osteocytes (Orriss et al., 2006; Plotkin et al., 2017). In osteoblasts mRNA for P2Y and P2X receptors were detected and it has been shown that their expression, as well as the amount of ATP released, depends on cellular differentiation status since osteoblasts' responsiveness to nucleotides increase in parallel with the cell differentiation and an increase of P2Y2 and P2X4 receptors was detected in mature osteoblasts compared to immature cells (Bowler et al., 1995; Maier et al., 1997; Hoebertz et al., 2000; Ihara et al., 2005; Orriss et al., 2006; Orriss et al., 2009; Orriss et al., 2012; Syberg et al., 2012). ATP release in osteoblasts is also linked to cell proliferation since it has been shown that activation of P2X5 receptor promotes DNA synthesis and P2Y2 increases the proliferation rate (Suzuki et al., 1993; Nakamura et al., 2000; Katz et al., 2008; Ayala-Pena et al., 2013). Also P2X7 receptor stimulation enhances mineralization of osteoblasts; defective osteogenesis in P2X7 KO mice leads to wider calvarial sutures (Panupinthu et al., 2007; Panupinthu et al., 2008; Manaka et al., 2015). In human osteoblasts and in rat osteosarcoma cell line, P2 receptors mediate intercellular propagation of the calcium wave (Jorgensen et al., 1997; Jorgensen et al., 2000). Moreover the calcium wave can propagate *in vitro* between osteoblasts and osteoclasts in a co-culture system, a mechanism involving the activation of the P2X7 receptor (Jorgensen et al., 2002). The effect on bone mineralization is instead controversial. Mechanical forces promote ATP release followed by RUNX2 activation in a human osteoblastic cell line (Costessi et al., 2005) but low concentrations of ATP inhibit mineralization by binding P2Y2 receptor and mice lacking this receptor show an increased bone volume (Orriss et al., 2007; Orriss et al., 2017). Clopidogrel, an inhibitor of platelet aggregation used as a therapy for secondary prevention of the stroke, is an antagonist of P2Y12 receptor and *in vitro* was able to inhibit murine osteoblast proliferation and viability and to reduce osteoblasts' ability to form bone nodules. Moreover, *in vivo*, clopidogrel reduces trabecular bone in femur and tibia of ovariectomized mice (Syberg et al., 2012). Nonetheless, in patients clopidogrel does not increase fracture risk (Jorgensen et al., 2017).

P2 receptors are also expressed in bone resorption cells (Burnstock et al., 2013). Through P2 receptors on osteoclasts, ATP induces a non-selective cation current (through P2X receptor) and the release of calcium from intracellular stores (P2Y receptors). The incubation of osteoclasts with suramin, a P2 receptor antagonist, blocks the generation of calcium waves (Wiebe et al., 1999). The P2Y12 receptor plays an important role in promoting osteoclast differentiation and activity. In models of arthritis P2Y12KO mice have increased trabecular bone and therefore are protected from osteopenia induced by arthritis, tumor growth in bone, and by ovariectomy (Su et al., 2012). Also

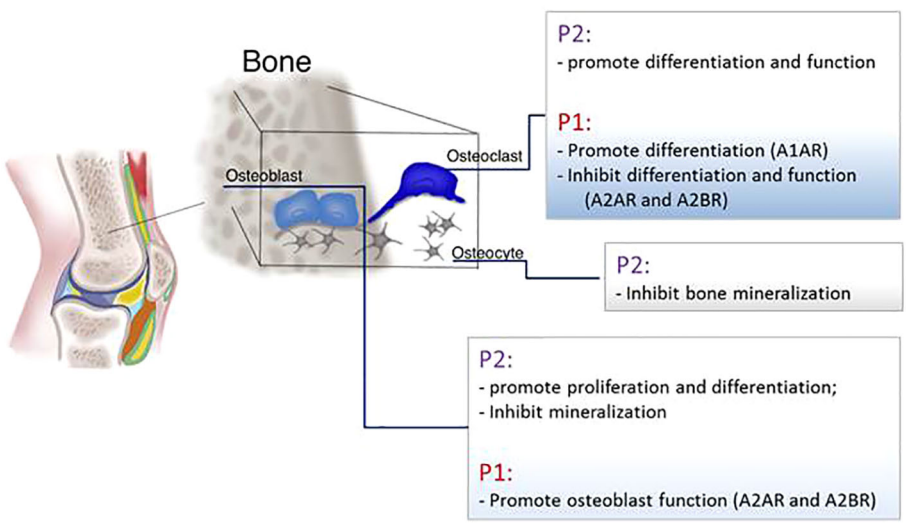

FIGURE 5 | Schematic representation of P1 and P2 receptors function in the main bone cells: osteoblasts, osteoclasts, and osteocytes.

in vitro, the active metabolite of Clopidogrel, a P2Y12 receptor antagonist, inhibits osteoclasts differentiation (Mediero et al., 2016). P2Y6 receptor has been associated to osteoclasts apoptosis, promotes NF-kB activation and osteoclast activity (Korcok et al., 2005; Lee et al., 2013). P2Y14, also, regulates osteoclast precursor differentiation by inducing RANKL (Lee et al., 2013). An extensive scientific literature is dedicated to exploring the effect of P2X7 receptor on bone resorption. Two clinical studies involving respectively 506 and 1,764 post-menopausal women, demonstrated that polymorphisms in the gene coding for the P2X7 receptors that diminish the binding of ATP to the receptors, contribute to the increased risk of lumbar spine fractures (Ohlendorff et al., 2007; Gartland et al., 2012). It is generally accepted that the P2X7 receptor is implicated in the generation of multinucleated giant cells and the *in vitro* administration of P2X7 receptor antagonists to human osteoclast precursors inhibits osteoclast differentiation (Gartland et al., 2003; Agrawal et al., 2010). Gartland et al. demonstrated that mice lacking P2X7 receptor do not exhibit important skeletal alterations whereas Ke et al. measured a reduction in cortical bone of the femur and an increase of the bone resorption in the tibia. Interestingly, both groups reported that osteoclast precursors isolated from these mice are still able to activate the process of cell fusion indicating an alternative path to differentiation that does not involve the P2X7 receptor (Gartland et al., 2003; Ke et al., 2003).

ATP is also released by osteocytes after mechanical stimulus and nucleotide stimulation with the effect of inhibiting bone mineralization in the surrounding area allowing the formation of lacunae (Genetos et al., 2007; Hajjawi et al., 2014; Kringelbach et al., 2014). Osteocyte apoptosis is a mechanism required for bone remodeling and precedes bone resorption; late-stage osteocytes produce RANKL in order to activate osteoclast differentiation. ATP plays a role in inducing osteocyte apoptosis through activation of P2X7 receptor in the intracortical area (Cheung et al., 2016).

In osteoblasts, UTP, but not UDP, increases ALP activity and expression of the osteogenic proteins BMP-2, 4, and 5 (Ayala-Pena et al., 2013). It has been shown that UTP and UDP facilitate the osteogenic differentiation of human bone marrow cells by P2Y6 receptor ligation (Noronha-Matos et al., 2012). At the same time, UDP through P2Y6 receptor stimulates the formation of osteoclasts from precursor cells and enhances their reabsorptive functionality. As a result of P2Y6 receptor role, mice lacking this receptor display more cortical bone (Orriss et al., 2011).

P1 Receptors

Patients with mutations in the ADA gene and the consequent increase in adenosine availability, present radiological bone defect including scapular and ribs changes, alteration due to imbalance between osteoclasts and osteoblasts functions resulting in low bone formation (Cederbaum et al., 1976; Sauer et al., 2009; Manson et al., 2013). mRNA of CD39 and CD73 is expressed in precursor and mature osteoclasts (He et al., 2013) and mice lacking CD73 have osteopenia associated with a higher number of osteoclasts compared to control mice and present a delay in bone regeneration due to an impairment in osteoblast activity (Takedachi et al., 2012; Bradaschia-Correa et al., 2017). Administration of Dipyridamole (440 mg/day), a drug that blocks adenosine re-uptake increasing its extracellular availability, was tested on RA patients and it has been shown that the drug does not raise blood purine levels and does not change patients symptoms (Forrest et al., 2006; Forrest et al., 2006). Instead, local administration of Dipyridamole by using 3D printed scaffolds was able to regenerate bone defects in mice and rabbits (Ishack et al., 2017; Conesa-Buendia et al., 2019; Witek et al., 2019; Lopez et al., 2019). These different outcomes could be explained by the rapid metabolism of adenosine in the bloodstream an effect avoided by the continuous and sustained local release mediated by the scaffold implant.

Adenosine promotes bone formation and resorption by activating different molecular pathways depending on which adenosine receptor is activated. Adult A1R KO mice show increased trabecular and cortical bone density compared to WT mice even if no difference in osteoblast number or osteoblast morphology has been measured (Kara et al., 2010). *In vitro*, the incubation of murine bone marrow and human osteoclasts precursors with A1R antagonists decreases their differentiation (Kara et al., 2010). For its effect, antagonism of A1R was studied in a post-ovariectomy osteoporosis mouse model and it has been shown that A1R blockade prevents bone loss primarily by inhibiting osteoclast differentiation without affecting osteoblast function and number (Kara et al., 2010). At the molecular level, the effect of A1R activation on osteoclasts is mediated by NF-kB and TRAF6 signaling leading to RANK activation (He and Cronstein, 2012).

With regard to the role of A2AR, *in vitro* studies gave contrasting results depending on what kind of osteoclast precursor has been used. Pellegatti and colleagues demonstrated that A2AR promotes osteoclasts differentiation from peripheral blood mononuclear cells facilitating osteoclast fusion (Pellegatti et al., 2011). In contrast, Mediero and colleagues demonstrated that activation of A2AR and cAMP activation reduces the number of differentiated osteoclasts isolated from murine bone marrow. These results were confirmed by the observation *in vivo* of A2ARKO mice showing increased osteoclast number and osteopenia (Mediero et al., 2015).

A2AR exerts also an indirect inhibitory effect on osteoclasts by blocking the production of pro-inflammatory cytokines like TNF-α and IL-1β leading to diminished RANKL production in an inflammatory setting (Kim et al., 2005; Chan and Cronstein, 2010; Bitto et al., 2011). Indeed, methotrexate treatment diminishes wear particle-induced osteolysis *via* adenosine ligation of A2AR (Mediero et al., 2015) and A2AR agonists reduce bone resorption and disease progression in an animal model of type II collagen-induced arthritis (Bitto et al., 2011; Mazzon et al., 2011). Similarly, the administration of dipyridamole, an agent that enhances extracellular adenosine by blocking ENT1-mediated adenosine uptake, promotes bone regeneration (Mediero et al., 2015; Mediero et al., 2016; Ishack et al., 2017). In our previous study we observed an osteopenic phenotype in A2BRKO mice and Carroll and colleagues

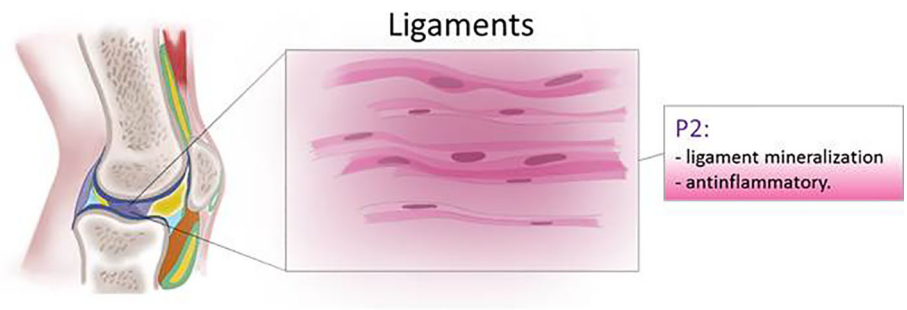

FIGURE 6 | Schematic representation of P2 receptor functions in ligaments.

demonstrated delayed fracture healing in this mouse strain compared to the WT. This phenotype has been ascribed to the inability of the murine A2BRKO cells to differentiate into osteoblasts and to enhanced osteoclast differentiation (Carroll et al., 2012; Corciulo et al., 2016). Similarly, human bone marrow stimulated with A2BR agonist diminishes osteoclasts differentiation (He et al., 2013).

No direct effect of A3R activation on bone has been reported. Moreover, neither the A3R activation nor blockade affects osteoclast differentiation *in vitro* (He et al., 2013). Instead, the effect of A3R on bone has been studied has a consequence of its anti-inflammatory function. In an arthritis rat model, the treatment with an A3R agonist reduces inflammation, bone destruction, and the number of osteoclasts in bone surface (Rath-Wolfson et al., 2006).

Ligaments and Tendons

Ligaments and tendons in the joint support and transmit mechanical loading to the musculoskeletal system by forming a dynamic connection between the skeletal bones and by linking muscle to bone. Injury or diseases of the connective tissue impair the mechanical function of ligaments and tendons mainly by reducing or changing their collagen composition (Zitnay and Weiss, 2018).

One of the diseases affecting the ligament is called ossification of the posterior longitudinal ligament spinal (OPLL) ligament due to ectopic bone formation of the spinal ligaments compressing the nerve root and causing neurologic problems. High levels of P2Y1 receptor expression and ATP release are responsible for osteoblastic differentiation of the cervical spinal ligament in patients affected by OPLL (Tanaka et al., 2011).

The role of the purinergic system in ligament function and structure has been largely studied in the periodontal tissues. In the periodontal ligament, P2Y4 and P2Y6 receptors are the most abundant P2Y receptors through which gravity released ATP induces phosphorylation of ERK and consequently collagen I and OPG release, essential for remodeling of the alveolar bone (Ito et al., 2014). In the human periodontal ligaments, the compression force promotes ATP release from the connexin 43 channel. P2Y receptor activation stimulates osteopontin and RANKL release contributing to the periodontal bone remodeling (Luckprom et al., 2010; Luckprom et al., 2011).

In vitro, elevated levels of ATP, typical in inflamed areas, increase apoptosis and caspase 3/7 expression of the periodontal ligament (Kawase et al., 2007). Periodontal ligaments, isolated from healthy volunteers, treated with the pro-inflammatory stimuli TNF-α or IL-1β showed a decreased expression of P2X7 receptor, the receptor associated with matrix mineralization and osteoblast differentiation (Xu et al., 2019).

Human tendon cells subjected to mechanical stretch release IL-1β, COX-2, and MMP-3 and ATP which has been proposed as a trigger of negative feedback to limit activation of the inflammatory pathway (Tsuzaki et al., 2003) (**Figure 6**).

CONCLUSION

In summary, extracellular purine nucleotides and their receptors are well established regulators of the viability and function of the tissues in the joint, playing an important role in maintaining homeostasis of the joint tissue cells. Stress and inflammation regulate the release of adenine and uridine nucleotides into the extracellular space as well as the enzymes that metabolize them. These interactions offer potential targets for interventions in diseases and conditions of the musculoskeletal system. Many attempts were made to target purine receptors to counteract joint damage in different rheumatic diseases, in human patients and in murine animal models, suggesting the efficacy of these drugs in counteracting inflammation. Nevertheless, since both P1 and P2 receptors are expressed on many different cells and organs it is likely that on-target toxicities will limit targeting of these receptors by systemic administration of agonists/antagonists. Local drug delivery of highly selective agonists and antagonists offer a promising way to make use of the beneficial effects of these agents.

AUTHOR CONTRIBUTIONS

CC: Literature search and drafting the manuscript. BC: revising and completion of the final work.

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Conflict of Interest: CC and BC have a patent for the use of adenosine and A2AR agonists for the treatment of OA. BC and CC are co-founders and own stocks in Regenosine Inc.

frontiers in Pharmacology

BRIEF RESEARCH REPORT
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The P2X7 Receptor 489C>T Gain of Function Polymorphism Favors HHV-6A Infection and Associates With Female Idiopathic Infertility

Anna Pegoraro[1†], Daria Bortolotti[2†], Roberto Marci[1,3], Elisabetta Caselli[2], Simonetta Falzoni[1], Elena De Marchi[1], Francesco Di Virgilio[1], Roberta Rizzo[2†] and Elena Adinolfi[1*†]*

[1] Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy, [2] Department of Medical Sciences, University of Ferrara, Ferrara, Italy, [3] Obstetrics and Gynaecology, School of Medicine, University of Geneve, Geneve, Switzerland

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***Correspondence:**
Roberta Rizzo
roberta.rizzo@unife.it
Elena Adinolfi
elena.adinolfi@unife.it

[†]These authors have contributed equally to this work

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The P2X7 receptor (P2X7R) is an ATP-gated ion channel known for its proinflammatory activity. Despite its participation in host defense against pathogens, the role played in viral infections, notably those caused by herpes viruses, has been seldom studied. Here we investigated the effect of P2X7R expression on human herpes virus 6 A (HHV-6A) infection of P2X7R-expressing HEK293 cells. We show that functional P2X7R increases while its blockade decreases viral load. Interestingly, HHV-6A infection was enhanced in HEK293 cells transfected with P2X7R cDNA bearing the gain of function 489C>T SNP (rs208294, replacing a histidine for tyrosine at position 155). The P2X7R 489C>T polymorphism correlated with HHV-6A infection also in a cohort of 50 women affected with idiopathic infertility, a condition previously shown to correlate with HHV-6A infection. None of the infertile women infected by HHV-6A was homozygote for 489CC genotype, while on the contrary HHV-6A infection significantly associated with the presence of the rs208294 allele. Levels of soluble human leukocyte antigen G (sHLA-G), a factor promoting embryo implant, measured in uterine flushings negatively correlated with the 489TT genotype and HHV-6A infection, while proinflammatory cytokines interleukins 1α (IL-1α), 1β (IL-1β), and 8 (IL-8) positively correlated with both the 489T allele presence and viral infection. Taken together these data point to the P2X7R as a new therapeutic target to prevent HHV-6A infection and the associated infertility.

Keywords: P2X7, P2X7 489C>T polymorphism, HHV-6A infection, female infertility, HLA-G

INTRODUCTION

The P2X7 receptor (P2X7R) ATP receptor is an ion channel belonging to the family of P2X receptors. The functional P2X7R is formed by a homomeric trimer whose subunit consists of a large extracellular loop including the agonist and antagonist binding sites, two alpha-helical transmembrane regions, and intracellular N and C-terminal peptides. Due to its widespread

expression on immune cells and its ability to release cytokines the best-recognized function of P2X7R is to participate in inflammatory reactions. In the inflammatory microenvironment, ATP is released from dying cells as a damage-associated molecular pattern (DAMP) to activate the P2X7R and the following NLRP3-dependent release of several proinflammatory cytokines including interleukin (IL)-1β, IL-1α, and IL-18 (Di Virgilio et al., 2017; Orioli et al., 2017; Adinolfi et al., 2018). Accordingly, P2X7R can down-modulate the activity of anti-inflammatory molecules such as human leukocyte antigen (HLA)-G (Rizzo et al., 2009), an HLA class Ib molecule implicated in embryo protection and implantation *via* immune cells inhibition (Rizzo et al., 2007b). Moreover, P2X7R promotes hypoxia-inducible factor 1-α (HIF-1α) activation, vascular endothelial growth factor (VEGF) secretion, and neovascularization (Hill et al., 2010; Adinolfi et al., 2012; Amoroso et al., 2012; Amoroso et al., 2015). The human P2X7R is a highly polymorphic gene harboring over 13.000 single nucleotide polymorphisms (SNPs) the majority of which are nonsynonymous, intronic, or missense (Benzaquen et al., 2019). However, a small number of these SNPs has been shown to change receptor function, either as loss- (10) or gain- (3) of-function variants (Stokes et al., 2010; Bradley et al., 2011; De Marchi et al., 2016; Sluyter, 2017). Such diversity has been attributed to environmental pressure by infectious agents, such as mycobacterium tuberculosis, and association with chronic inflammatory diseases (Adinolfi et al., 2018). The most frequent human P2X7R SNP is 489C>T (rs208294, 47% frequency), leading the substitution of histidine 155, localized in the P2X7R ectodomain, with a tyrosine (H155Y) (Cabrini et al., 2005). Intriguingly, the CC genotype seems to be present mainly in the human receptor while the TT genotype, is the prevailing variant of the receptor in both mouse and rat (North and Surprenant, 2000). The 489C>T gain of function polymorphism has been recently associated with Alzheimer's disease pathogenesis (Sanz et al., 2014) and in the accelerated release of proinflammatory cytokines observed in Lupus complicated with pericarditis (Hu et al., 2019). However, its implication in human disorders etiology remains understudied.

The P2X7R plays a major role in the response to infectious diseases, in particular, those caused by intracellular pathogens, *via* either direct effect on pathogen cell entry and survival or modulation of innate and adaptive immune responses (Adinolfi et al., 2018; Savio et al., 2018; Savio and Coutinho-Silva, 2019). In this context, the effect of P2X7R activation is not always beneficial or detrimental but depends upon the specific pathogen, its virulence and the severity of the infection (Savio et al., 2018). Therefore, the P2X7R can act as both host-protecting and infection-promoting factor. In viral infections, P2X7R inhibition protects against hepatitis, influenza, adenoviruses and HIV but it is detrimental in the case of vesicular stomatitis and Dengue viruses (Adinolfi et al., 2018; Savio et al., 2018). As per herpes viruses infections, limited evidence is available, showing P2X7R upregulation following cytomegalovirus infection (Zandberg et al., 2007). Human herpes virus 6 (HHV-6), a member of the Betaherpesvirinae subfamily and the causative agent of roseola infantum, has a wide cell tropism, albeit T cells are a preferred target. The acronym HHV-6 includes two distinct viruses, HHV-6A and -6B. HHV-6A recently emerged as a possible determinant of female idiopathic infertility, because infection by this virus alters endometrial immune cell responses and cytokine composition of the uterine milieu (Marci et al., 2016; Caselli et al., 2017; Bortolotti et al., 2019). Furthermore, HHV-6A infection causes a reduction in protein levels of the widely used phenotypic decidualization markers HLA-G (Rizzo et al., 2011) and mucin1 (MUC1) (McAuley et al., 2017). In this study, we explored the role played by the P2X7R in favoring HHV-6A infection and the associated infertility.

MATERIALS AND METHODS

Cell Lines and Cultures

The acronyms HEK CC, HEK TT, and HEK mock are referring to HEK293 cells stably expressing human P2X7R-489CC, human P2X7R-489TT, and the empty expression vector PcDNA3, which were obtained as described in (Adinolfi et al., 2005; Cabrini et al., 2005). HEK293 cells were cultured in DMEM high glucose (Sigma), complemented with 10% heat-inactivated fetal bovine serum (FBS) (Euroclone, Milan, Italy), 100 U/ml penicillin (Euroclone), 100 mg/ml streptomycin (Euroclone), 1% non-essential amino acids (Sigma), and G418 sulfate 0,4 mg/ml (Sigma). HEC-1A endometrial epithelial cancer cell line (ATCC HTB-112) was cultured in McCoy's 5a Medium (Sigma).

P2X7R Immunofluorescence

Receptor's surface expression was confirmed by immunocytochemistry (see **Supplementary Figure 1**). HEK mock, HEK CC and HEK TT cells were seeded on coverslips fixed with 4% paraformaldehyde for 15 min at 37°C, rinsed three times with phosphate-buffered saline (PBS), incubated in blocking buffer [PBS with 1% bovine serum albumin (BSA)] for 45 min at room temperature. Coverslips were then incubated with the antihuman P2X7R monoclonal antibody (mAb), previously characterized by Buell et al. (1998) kindly provided by Professor James Wiley (Florey Neuroscience Institutes, University of Melbourne, Australia) overnight at 4°C at a dilution of 1:50. Samples were rinsed and incubated with a TRICT conjugated anti-mouse antibody (T5393, Sigma-Aldrich) at a dilution of 1:500 for 1 h at room temperature. Fluorescence was visualized with a Leica DMI 4000B microscope equipped with 100× oil objective and images were acquired thanks to a Leica DFC 550 camera.

HHV-6A Cell Infection and Assays for HHV-6 Detection

HEK293 and HEC-1A cells were infected with HHV-6A (strain U1102) cell-free virus inocula (Caselli et al., 2017) at a multiplicity of infection of 100 genome equivalents per 1 cell for 2 h at 37°C. HHV-6A antigen expression was analyzed by immunofluorescence 5 days post-infection (dpi) with a mouse

mAb that recognized the glycoprotein gp116 (late antigen) of HHV-6A (ABI, Columbia, MD, United States), as previously described (Caselli et al., 2017).

HHV-6A DNA extraction and analysis was performed as previously described (Rizzo et al., 2019). Briefly, real-time quantitative polymerase chain reaction (qPCR) specific for the U94 gene was used to determine HHV-6A DNA presence and load. Positive samples were considered those in which 1 μg of cellular DNA harbored more than 100 copies of viral DNA. The following set of primers/probe was used for qPCR: HHV6 U94 (+) (5′-GAG CGC CCG ATA TTA AAT GGA T-3′); HHV6 U94 (−) (5′-GCT TGA GCG TAC CAC TTT GCA-3′); HHV6 U94 PROBE (5′-FAM-CTG GAA TAA TAA AAC TGC CGT CCC CAC C-TAMRA-3′). The standard curve was generated by amplification of a plasmid containing the targeted HHV-6 sequences. Human RNase P or beta-actin housekeeping genes were used as a control.

RNA cell extraction was performed with the RNeasy kit (Qiagen, Hilden, Germany). The absence of contaminant DNA in the extracted RNA was assured by DNase treatment and control β-actin PCR without retrotranscription reverse transcription (Caselli et al., 2017; Rizzo et al., 2017). The analysis of virus transcripts was performed by RNA reverse transcription with the RT2 First-strand kit (Qiagen, Hilden, Germany) using cDNA aliquots obtained from 200 ng RNA (Menegazzi et al., 1999; Caselli et al., 2012). The specific primers used to amplify HHV-6A U42 were: forward 3′ACGATGGA CATGGCTTGTTG5′; reverse 3′ACCTTACAACGGAGAC GCC5′ (Caselli et al., 2012). The methods had a 6-log dynamic range and a sensitivity of 20 copies/ml. Each sample was run in duplicate.

HLA-G Detection

Ten microliters of cell culture supernatants or uterine flushing samples were assayed for soluble HLA-G using a bead array Bio-Plex system (BioRad, CA, USA) with anti-HLA-G MoAb (G233; Exbio, Czech Republic) conjugated beads, as previously reported (Rizzo et al., 2007a).

CD46 Flow Cytometry

CD46 surface expression data were analyzed using FACS CantoII flow cytometer (BD, Milan, Italy) and FlowJo LLC analysis software (Ashland, Oregon, USA). Cells (1×10^5) were labeled with CD46-PE (R&D Systems, Italy) or matched isotype controls. Ten thousand events per cell type were acquired.

Clinical Samples

Endometrial specimens were obtained by biopsy from patients admitted for tubal patency assessment by Hystero-sono contrast sonography at secretory stage of the menstrual cycle. The endometrium was prepared as previously described (Marci et al., 2016). Endometrial epithelial cells were collected from the Ficoll-Paque-medium interface using BerEP4-coated magnetic Dynabeads system (Dynal Biotech, Oslo, Norway). The sorted epithelial cells were typed for HHV-6A or B identification. DNA extraction and analysis were performed as previously described (Caselli et al., 2012). Above-mentioned qPCR was used to determine HHV-6 DNA presence and load. All samples were randomly and blindly investigated and we obtained enough material the analysis was repeated twice in a randomized and blinded fashion at a distant time set from the first determination. HHV-6A or B identification was performed as reported previously (Caselli et al., 2012), by restriction enzyme digestion of the U31 nested PCR amplification product and visualization of the digestion products on ethidium bromide-stained agarose gel after electrophoresis migration.

Uterine flushing samples were obtained from the same patients including only 21–38 years old women, with regular menstrual cycle (24–35 days), a body mass index (BMI) ranging between 18 and 26 kg/m^2, follicle-stimulating hormone (FSH; days 2–3 of the menstrual cycle) < 10 mUI/ml, 17-β-estradiol < 50 pg/ml (days 2–3 of the menstrual cycle), normal karyotype. Women that presented endometritis, endometriosis, tubal factor, ovulatory dysfunction, anatomical uterine pathologies, and recurrent miscarriage were excluded. Uterine flushing was performed with a 14-gauge Foley three-way balloon catheter (Eschmann) inflating an appropriate (5 ml) amount of sterile physiologic saline solution (Rizzo et al., 2017). Genomic DNA for P2X7R polymorphism assessment and circulating HHV6-A and B detection was isolated from whole blood using the QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany). This study was approved by the "University-Hospital of Ferrara Ethics Committee." All subjects gave written informed consent in accordance with the Declaration of Helsinki.

P2X7R 489C>T Polymorphism Analysis

The presence of 489C>T SNP was determined by real-time PCR using an allelic discrimination TaqMan MGB probe technique with a Step One Real-Time PCR thermal cycler (Applied Biosystem) as previously described (Cabrini et al., 2005). Briefly, genomic DNA (100 ng) was added to the PCR master mix (TaqMan Universal PCR Master Mix, Applied Biosystems) in the presence of validated primers and probes (Applied Biosystems, ID number: C:_3019032_1_). Following 40 PCR cycles (15 s at 95°C for denaturation and 1 min at 60°C annealing plus elongation), genotype was assigned to each sample analyzing the fluorescent signal.

Cytokines and Growth Factor Evaluation in Uterine Flushing Samples

Cytokines were analyzed in uterine flushing samples using Cyraplex assay (Aushon, distributed by Tema Ricerca, Bologna, Italy) according to the manufacturers' instructions. VEGF levels were assessed by PicoKine™ ELISA kit (Boster, distributed by Tema Ricerca, Bologna, Italy).

Measurement of Cytosolic Ca^{2+}

Changes in the intracellular HEC-1A Ca^{2+} concentration were measured with the fluorescent indicator Fura-2/acetoxymethylester (Fura-2/AM), using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Milan, Italy). Cells were seeded on a coverslip and maintained at 37°C in a 5% CO_2 humidified incubator until confluence. The confluent monolayers were loaded with Fura-2/AM (4 μM) in standard

saline solution: 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 20 mM HEPES, 5.5 mM glucose, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, and 250 μM sulfinpyrazone (Sigma-Aldrich). Incubation was performed at 37°C for 20 min. Cells were then washed and changes of intracellular [Ca^{2+}] were determined after stimulation with 300 μM 2′-(3′)-0-(4-benzoylbenzoyl) ATP (Sigma-Aldrich) following, whenever required, by 10-min incubation with AZ10606120, in a thermostated cuvette under stirring, with a 340/380 excitation ratio at an emission wavelength of 505 nm.

Statistics and Data Availability

Data were analyzed for normality by Kolmogorov–Smirnov test. All the variables resulted normally distributed. Therefore, analysis of variance (ANOVA) and two-tailed Student's t tests were used to compare most of the variables. X^2 test and Fisher exact test were used to compare allelic and genotypic frequencies and positivity for HHV6-A (**Table 1**) and sHLA-G (**Figure 2B**) respectively. P-values lower than 0.05 were considered statistically significant. All statistic evaluations were performed thanks to GraphPad Prism software (GraphPad, La Jolla, California, USA). The raw data supporting the conclusions of this manuscript will be made available by the authors, upon reasonable request, to any qualified researcher.

RESULTS

P2X7R Favors HHV-6A Infection in HEK293 Cells

HEK293 human embryonic kidney cells are an interesting model for P2X scholars as they do not express a functional P2X7R receptor or any other member of the P2X family and can be easily transfected to induce the overexpression of virtually all P2X receptors (Adinolfi et al., 2005). Therefore we used HEK293 stably transfected cell clones carrying either a) the WT P2X7R SNP, i.e. the 489CC genotype (HEK CC), or b) the gain of function P2X7R SNP, i.e. the 489TT genotype (HEK TT) or the empty vector (HEK mock) and challenged them with HHV-6A virions. **Figure 1** shows that P2X7R expression strongly increases HHV-6A infection in HEK293 cells. The viral infection is dependent upon the 489 P2X7R genotype as HEK TT are infected in a significantly higher percentage if compared to HEK CC (**Figures 1A, B, F**). Moreover, both late gene U94 DNA and immediate early U42 gene RNA levels are 1 log higher in HEK TT in comparison with HEK CC and the expression of both receptors variants leads to an increase of about 2 logs of HHV6-A genes as compared to HEK mock cells (**Figures 1G, H**). P2X7R antagonism with AZ10606120 strongly reduced HHV-6A infection in HEK TT cells as demonstrated by both immunofluorescence (**Figures 1B, E, F**) and gene evaluation data (**Figures 1G, H**). However, HEK CC HHV-6A positivity was only slightly reduced by the receptor antagonist (**Figures 1G, H**) and viral DNA and RNA expression, although presenting a clear trend toward reduction (~20%) were not significantly affected (**Figures 1G, H**). Since HHV-6A entry into target cells is determined by CD46 surface expression (Santoro et al., 1999), we evaluated CD46 levels on the surface of HEK293 transfected with the different P2X7R variants (**Figure 1I**). However, P2X7R expression or genotype did not affect the expression of the HHV-6A receptor CD46 (**Figure 1I**) ($p = 0.34$; ANOVA test), supporting a role for P2X7R in the control of viral infection. Since we know that both P2X7R and HHV-6 can affect HLA-G expression (Rizzo et al., 2009; Caselli et al., 2015), we measured HLA-G concentration in HEK293 culture supernatants (**Figure 1J**). Secretion of HLA-G was different between the different samples ($p = 0.0012$; ANOVA test). Soluble HLA-G (sHLA-G) inversely correlated with P2X7R expression and was dependent upon 489 SNP with the 489TT genotype associated with a larger reduction of HLA-G release ($p < 0.001$; Student t-test) (**Figure 1J**).

P2X7R 489CC Protects Against HHV-6A Infection and Affects the Uterine Cytokine Milieu in Infertile Women

We next investigated the association of P2X7R 489C>T SNP with HHV-6A infection in a pathological condition. HHV-6A infection and low HLA-G levels in the uterine environment were recently associated with female idiopathic infertility (Marci et al., 2016; Bortolotti et al., 2019), therefore we analyzed the frequency of 489C>T SNP and the presence of HHV-6A DNA in endometrial biopsies from a cohort of 50 women with idiopathic infertility. The endometrial biopsies were analyzed for the presence of HHV-6A and B infection. More than 20% (11/50) women with idiopathic infertility were positive for HHV-6A DNA in their endometrial epithelial cells. HHV-6B DNA was not present in all the endometrial biopsies as previously reported (Marci et al., 2016; Bortolotti et al., 2019). The average viral load in endometrial epithelial cells from HHV-6A positive infertile women was 490.000 copies/μg of cellular DNA (range 698.000–236.000 copies/μg DNA), corresponding to about 4 copies of viral DNA per diploid cell. As a control, we evaluated HHV-6A and HHV-6B DNA presence in the peripheral blood mononuclear cells (PBMCs) of our population. While we confirmed the previously reported 25% positivity for HHV-6B virus (Caselli, 2007) HHV6-A was not present in PBMCs from our population (not shown). **Table 1** shows a negative association between HHV-6A infection and 489 C homozygosis with the number of infected women significantly growing with the presence of the minor gain of function allele 489T. These data strongly suggest an association between the rs208294 allele and the susceptibility to HHV6-A infection in idiopathic infertile women. Analysis of HLA-G concentration in uterine flushings from the same women revealed a clear tendency toward a decrease of HLA-G from HHV-6A negative/489CC to HHV-6A positive/489TT subjects (**Figure 2A**). The high variability in HLA-G concentration found in our population was due to the presence of flushing samples where HLA-G was either absent or below the threshold for detection. Therefore, we re-analyzed the population comparing the percentage of HLA-G positive versus HLA-G negative flushing samples by fisher exact test (**Figure 2B**). This analysis highlighted statistically significant

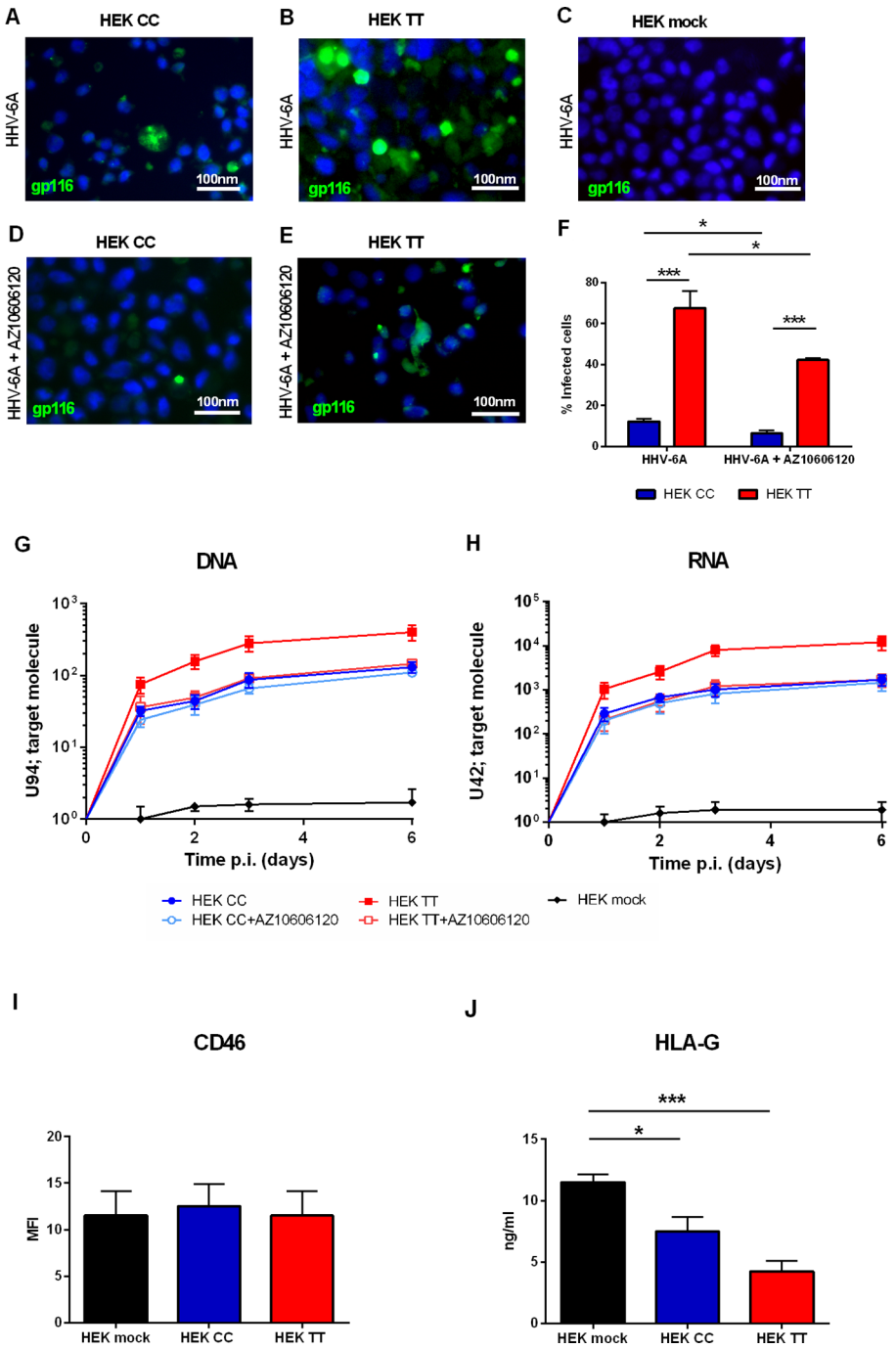

FIGURE 1 | (A–H) HHV6-A infection of HEK293 cells. Cells were infected with 100 genome equivalents per cell of HHV-6A for 2 h at 37°C in the presence or not of P2X7R antagonist AZ10606120 (2 μM). **(A–E)** Fluorescence pictures of HHV-6A infected HEK CC **(A, D)**, HEK TT **(B, E)**, and HEK mock cells **(C)** stained with anti-gp116 mAb. **(F)** Percentage of HEK CC and HEK TT cells positive for HHV-6A gp116 protein in the presence or not of P2X7R antagonist AZ10606120. HHV-6A positivity significantly differs among groups [$p < 0.0001$, analysis of variance (ANOVA) test]. Data for each group are shown as the mean ± SEM of four experiments. Groups were compared with each other using two-tailed Student's t-test, $^{*}p < 0.05$, $^{***}p < 0.001$. HHV6-A U94 DNA **(G)** and U42 RNA levels **(H)**. U94 DNA and U42 RNA were quantified by polymerase chain reaction (PCR) and quantitative PCR (qPCR) as reported in *Materials and Methods*. **(I)** Mean fluorescence intensity (MFI) of HHV-6A membrane receptor CD46 on HEK mock, HEK CC, and HEK TT cells. Expression of CD46 is not significantly different among groups ($p = 0.34$, ANOVA test). **(J)** Human leukocyte antigen G (HLA-G) concentration was evaluated in HEK mock, HEK CC, and HEK TT cell culture supernatants and differed among the samples ($p = 0.0012$; ANOVA test). Data for each group are shown as the mean ± SEM of four experiments. Groups were further compared with each other using two-tailed Student's t-test, $^{*}p < 0.05$, $^{***}p < 0.001$.

TABLE 1 | Allele and genotype frequencies of P2X7R polymorphism 489C>T in infertile women population (n = 50) subdivided according to HHV-6A infection into HHV-6A negative women (HHV-6A–, n = 39) and HHV-6A positive women (HHV-6A+, n = 11).

Polymorphism 489C>T	Allele frequency %		Genotype frequency %		
	C	T	CC	CT	TT
Infertile women* (n = 50)	0.58	0.42	32	52	16
HHV-6A infection					
HHV-6A -$ (n = 39)	0.65	0.35	41	49	10
HHV-6A +*$ (n = 11)	0.32	0.68	0	63	37

*$p = 0.0358$ HHV-6A+ women vs overall population (X^2 test).
$^{\$}p = 0.0021$ HHV-6A - women vs HHV-6A+ women (X^2 test).
The genotype frequencies of HHV-6A– and HHV-6A+ women were compared between them and with those of the total tested population. The genotype frequencies were found significantly different in HHV-6A+ women as compared to the overall population (p = 0.0358) and HHV-6A– women (p = 0.0021). While, no significant difference between HHV-6A– women and the overall population was found (p = 0.3911). P-value was calculated with the X^2 test. HHV-6A negative with P2X7R 489 genotype CC (n = 16), HHV-6A negative with P2X7R 489 genotype CT (n = 19), HHV-6A negative with P2X7R 489 genotype TT (n = 4), HHV-6A positive with P2X7R 489 genotype CT (n = 7), HHV-6A positive with P2X7R 489 genotype TT (n = 4).

differences between women clustered according to the 489 genotype and HHV-6A status of infection, with the HHV-6A negative/489CC subjects showing the highest HLA-G percentages as compared to infected and 489TT women (**Figure 2B**). On the contrary, proinflammatory cytokines and chemokines showed a tendency to increase in the presence of 489T allele and HHV-6A infection (**Figures 2C–E**). As expected, based on the central role played by P2X7R in its maturation and secretion, IL-1β flushing concentrations differed between the cohorts (p = 0.0132; ANOVA test), tending to increase in women with the 489T allele, reaching statistically significantly high concentrations in subjects both homozygous for 489T and infected with HHV-6A ($p < 0.001$; Student t-test) (**Figure 2C**). A similar trend was also observed for IL-1α (p = 0.03; ANOVA test) (**Figure 2D**) and IL-8 (p = 0.045; ANOVA test) (**Figure 2E**). Finally VEGF concentration in the flushing samples solely associated with 489TT genotype independently of the infection status (p = 0.012; ANOVA test) (**Figure 2F**). Other cytokines tested, including IFNγ, IL-4, IL-6, IL-10, IL-12p70, and TNF-α, were either undetectable or present at low concentrations and did not significantly differ within the tested populations (**Supplementary Figure 2**). As a proof of concept of the expression of P2X7R by endometrial epithelial cells, we analyzed HEC-1A endometrial epithelial cell line. Interestingly, a functional P2X7R, homozygous for the 489T allele, was also found in these cells (**Figure 3A**). We observed significant infection of these cells that was reduced by P2X7R antagonism with AZ10606120 (**Figure 3B**).

DISCUSSION

Purinergic signaling is known to participate in viral infection and replication, and in anti-viral inflammatory responses and, consequently, purinergic receptors have been proposed as targets for antiviral therapy (Ferrari et al., 2018; Savio et al., 2018; Zhang et al., 2019). Among P2 purinergic receptors, the P2X7R is one of the best candidates for such intervention as its activity in promoting or inhibiting viral infection, or alternatively its ability to support antiviral responses was widely demonstrated. Furthermore, several potent and selective inhibitors have been developed over time (De Marchi et al., 2016; Di Virgilio et al., 2017; Adinolfi et al., 2018; Savio et al., 2018). In the present study, we showed a predisposing role for the P2X7R in mediating HHV-6A infection. This activity was not due to changes in the expression of the known viral receptor CD46 since its levels remained unaltered in P2X7R expressing cells as compared to mock control. In support of a main role of a functional P2X7R, we also showed that a selective P2X7R blocker, AZ10606120 (De Marchi et al., 2019), reduced HHV-6A infection especially in HEK TT P2X7R-expressing cells. The identification of new drugs that can counteract HHV-6 infection is of extreme importance. Increased awareness of diseases associated with HHV-6 acute infection, in both immunocompetent and immunocompromised patients, has spurred interest in the development of effective treatments for HHV-6-mediated diseases. A number of drugs used for cytomegalovirus infection have shown *in vitro* efficacy against HHV-6. Dozens of case reports and several small clinical studies have demonstrated the efficacy of different compounds against HHV-6 infection. However, no drug has yet been approved for use in humans. The discovery that P2X7R expression facilitates HHV-6A infection opens an entirely novel avenue for anti-viral drug therapy. Our data show that HHV-6A infection *in vitro* and *in vivo* depends on P2X7R function as it is enhanced in the presence of the gain of function 489TT polymorphic variant. Previous studies showed that this polymorphism increases P2X7R activity as both a small ion channel and large cation permeation pathway (Cabrini et al., 2005; Roger et al., 2010) and that this phenotype is possibly linked to increased cell surface expression of P2X7R (Bradley et al., 2011). These data suggest that the SNP could favor viral entry either due to P2X7R membrane pore formation or to viral direct interaction on the cell surface. Another possible explanation for P2X7R-mediated HHV6-A cellular entry could be receptor-dependent phagocytic uptake (Gu and Wiley, 2018; Ou et al., 2018). Moreover, P2X7R-dependent increased HHV-6A infection inversely correlated with the secretion of HLA-G from HEK293 cells thus suggesting also an effect on the activation of the immune system (Eliassen et al., 2017). Data from HEK293 cells were confirmed by the complete absence of the 489CC homozygous genotype in primary infertile women bearing an endometrial HHV-6A infection (**Table 1**). To further support the role of the T allele in promoting HHV-6A infection, infection rates increase in parallel with 489T allele (**Table 1**). In agreement with the findings in HEK293 cells, also in the patient cohort the 489TT genotype correlated with decreased presence of sHLA-G in uterine flushing samples (**Figures 2A, B**). This observation is in keeping with the known modulatory effect of HHV-6A infection on HLA-G expression (Rizzo et al., 2018). HHV-6-induced increase of HLA-G is likely mediated by human

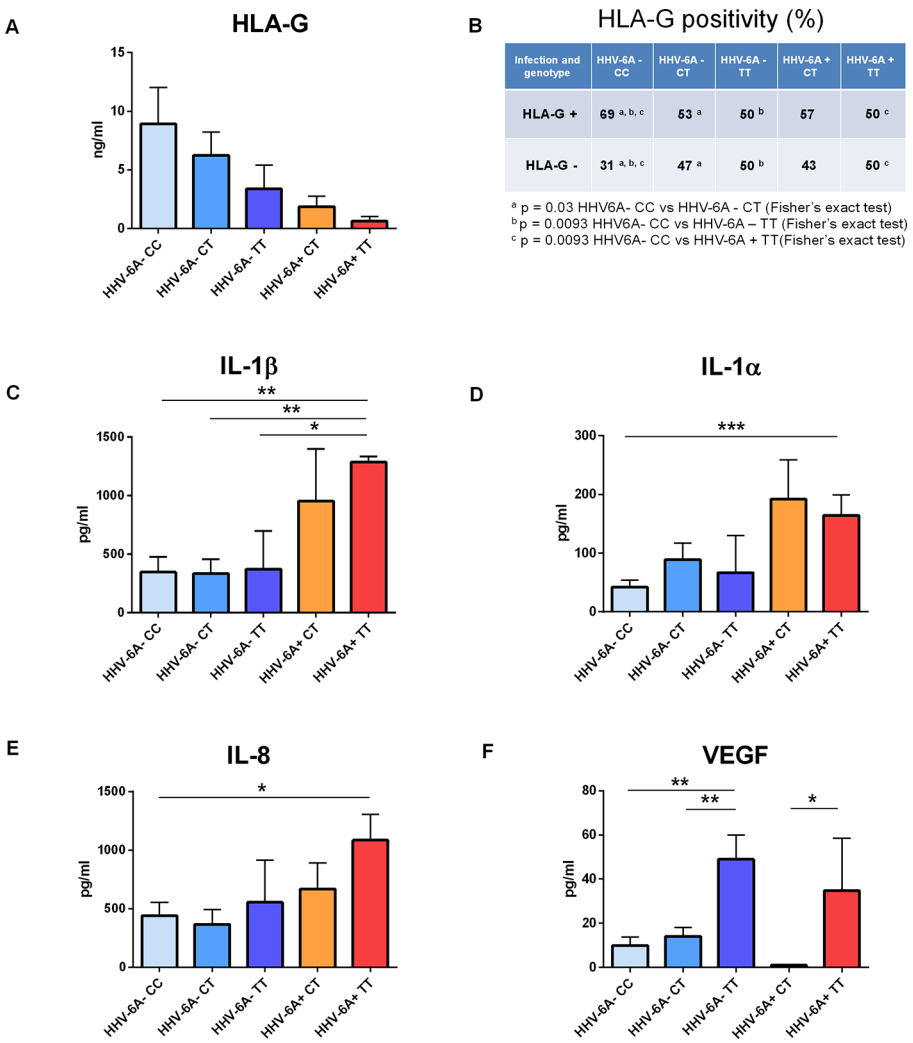

FIGURE 2 | Correlation between P2X7R polymorphism 489C>T and uterine milieu composition of infertile women infected or not with HHV-6A virus. **(A–F)** HLA-G, interleukin (IL)-1β, IL-1α, IL-8, and vascular endothelial growth factor (VEGF) concentrations were evaluated in uterine flushing samples from infertile women (n = 50) and analyzed according to P2X7R 489 genotype and HHV-6A infection as follows: HHV-6A negative with P2X7R 489 genotype CC (n = 16), HHV-6A negative with P2X7R 489 genotype CT (n = 19), HHV-6A negative with P2X7R 489 genotype TT (n = 4), HHV-6A positive with P2X7R 489 genotype CT (n = 7), and HHV-6A positive with P2X7R 489 genotype TT (n = 4). **(A)** HLA-G concentrations are not significantly different among the groups analyzed (p = 0.3196, ANOVA test). However, when analyzing the percentage of samples positive for HLA-G **(B)** according to P2X7R 489C>T polymorphism and HHV-6A infection there is a significant decrease of positivity of HLA-G in women positive for the virus and carrying 489T allele **(B)** ($p < 0.05$, Fisher's exact test). **(C–F)** Proinflammatory cytokines and VEGF concentrations are affected by P2X7R genotype and HHV-6A positivity. IL-1β **(C)**, IL-1α **(D)**, IL-8 **(E)**, and VEGF **(F)** concentrations are different among the samples by ANOVA test (p = 0.0132, p = 0.03, p = 0.045, p = 0.012, respectively). Data for each group are shown as the mean ± SEM and compared with each other using two-tailed Student's t-test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

transcription factor ATF3, that binds a consensus sequence on the HLA-G promoter (Rizzo et al., 2018). The largest difference in cytokines, chemokines and growth factors in uterine flushings was measured between the 489 CC/HHV-6A negative and the 489TT/HHV-6A positive subjects. 489TT/HHV-6A positive women also showed higher levels of IL-1β, IL-1α, and IL-8 (CXCL8) suggesting that the 489TT gain-of-function variant not only favors virus entry but also triggers a wide inflammatory response that might contribute to female infertility (Tsimis et al., 2017). It is therefore tempting to speculate that P2X7R-mediated HHV-6A infection and the associated female-infertility might have been a drive for negative selection of the 489T variant in favor of the 489C variant, and a possible mechanism that has favored this hypomorphic allele in humans as opposed to rodents, which are not infected by HHV-6 (Reynaud and Horvat, 2013) and where the 489T is the common allele (Surprenant et al., 1996; Rassendren et al., 1997; Chessell et al., 1998). Our data also confirm that the 489T allele increases P2X7R channel and pore function (Cabrini et al., 2005; Roger et al., 2010) as well as IL-1, IL-8, and VEGF secretion

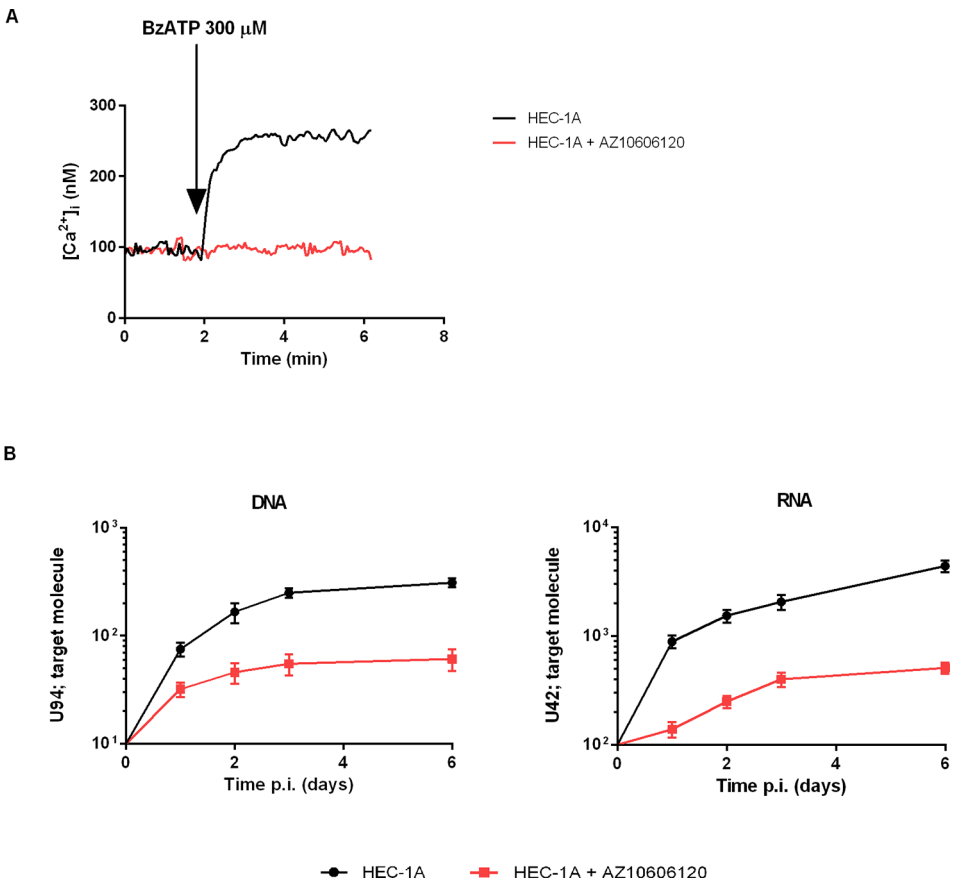

FIGURE 3 | Demonstration of functional P2X7R in the endometrial cancer cell line HEC-1A and infection with HHV-6A virus. HEC-1A carries the P2X7R 489TT genotype. **(A)** Representative trace showing P2X7R-dependent intracellular calcium increase following cells stimulation with BzATP (300 μM) alone (black) or in the presence of P2X7R antagonist AZ10606120 (2 μM, red). **(B)** HHV-6A infection in HEC-1A cells. Cells were infected with 100 genome equivalents per 1 cell of HHV-6A for 2 h at 37°C. U94 DNA and U42 RNA were quantified by real-time PCR and RT-PCR as reported in *Materials and Methods*. Black control cells, red cells pretreated with 2 μM AZ10606120.

(Orioli et al., 2017). P2X7R-mediated modulation of the uterine microenvironment could be due to both immune and endometrial cells, as these latter do express a functional P2X7R. Interestingly, P2X7R antagonism strongly reduced HHV6-A infection rate in HEC-1A endometrial cells (**Figure 3**) opening the way to the application of P2X7R targeting drugs also to this cellular subset.

Our data suggest a possible therapeutic application of P2X7R antagonists in young idiopathic infertile women. P2X7R blockers could be ideally locally administered in the endometrium to prevent or lower HHV-6A infection. Additionally, P2X7R blockade during the implantation window should help to reconstitute endometrial receptivity on one hand *via* induction of HLA-G and on the other *via* reduction of proinflammatory cytokines, chemokines, and VEGF. The recent use of P2X7R antagonists in several clinical trials guarantees their safety and a quick transfer to the clinical practice (De Marchi et al., 2016; Di Virgilio et al., 2017; Park and Kim, 2017; Di Virgilio et al., 2018), making them in principle useful therapeutic tools also in other HHV-6A-associated diseases such as roseola infantum, autoimmune diseases, chronic fatigue syndrome, encephalitis, and Alzheimer's disease (Rizzo et al., 2019).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of the manuscript will be made available by the authors, upon reasonable request, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University Hospital of Ferrara Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AP and DB performed most of the experimental work, helped with paper writing and experimental design. RM was responsible for patients selection and clinical evaluation. SF and EDM helped with PCR and *in vitro* experiments. EC helped with viral infections and experimental design. FDV participated in experimental design and manuscript writing. EA and RR were responsible for overall study conception, experimental design, data interpretation, manuscript writing, and final approval. AP and DB equally contributed to the study. EA and RR equally contributed to this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00096/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | P2X7R surface expression determined by immunofluorescence with anti-P2X7R mAb in **(A)** HEK mock, **(B)** HEK CC and **(C)** HEK TT.

SUPPLEMENTARY FIGURE 2 | (A) IFN-γ, **(B)** IL-4, **(C)** IL-6, **(D)** IL-10, **(E)** IL-12p70 and **(F)** TNF-α concentrations were evaluated in uterine flushing samples from infertile women (n = 50) and analyzed according to P2X7R 489 genotype and HHV-6A infection as follows: HHV-6A negative with P2X7R 489 genotype CC (n = 16), HHV-6A negative with P2X7R 489 genotype CT (n = 19), HHV-6A negative with P2X7R 489 genotype TT (n = 4), HHV-6A positive with P2X7R 489 genotype CT (n = 7), HHV-6A positive with P2X7R 489 genotype TT (n = 4). They did not significantly differ within the tested populations by ANOVA test (p = 0.2503, p = 0.7628, p = 0.2436, p = 0.6017, p = 0.7609, p = 0.5105, respectively).

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REVIEW
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P2 Receptors as Therapeutic Targets in the Salivary Gland: From Physiology to Dysfunction

*Mahmoud G. Khalafalla[1,2,3], Lucas T. Woods[1,2], Kimberly J. Jasmer[1,2], Kevin Muñoz Forti[1,2], Jean M. Camden[1,2], Janicke L. Jensen[4], Kirsten H. Limesand[5], Hilde K. Galtung[6] and Gary A. Weisman[1,2]**

[1] Department of Biochemistry, University of Missouri, Columbia, MO, United States, [2] Christopher S. Bond Life Sciences Center, University of Missouri, Columbia, MO, United States, [3] Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL, United States, [4] Institute of Clinical Dentistry, Section of Oral Surgery and Oral Medicine, University of Oslo, Oslo, Norway, [5] Department of Nutritional Sciences, University of Arizona, Tucson, AZ, United States, [6] Institute of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway

Although often overlooked in our daily lives, saliva performs a host of necessary physiological functions, including lubricating and protecting the oral cavity, facilitating taste sensation and digestion and maintaining tooth enamel. Therefore, salivary gland dysfunction and hyposalivation, often resulting from pathogenesis of the autoimmune disease Sjögren's syndrome or from radiotherapy of the head and neck region during cancer treatment, severely reduce the quality of life of afflicted patients and can lead to dental caries, periodontitis, digestive disorders, loss of taste and difficulty speaking. Since their initial discovery in the 1970s, P2 purinergic receptors for extracellular nucleotides, including ATP-gated ion channel P2X and G protein-coupled P2Y receptors, have been shown to mediate physiological processes in numerous tissues, including the salivary glands where P2 receptors represent a link between canonical and non-canonical saliva secretion. Additionally, extracellular nucleotides released during periods of cellular stress and inflammation act as a tissue alarmin to coordinate immunological and tissue repair responses through P2 receptor activation. Accordingly, P2 receptors have gained widespread clinical interest with agonists and antagonists either currently undergoing clinical trials or already approved for human use. Here, we review the contributions of P2 receptors to salivary gland function and describe their role in salivary gland dysfunction. We further consider their potential as therapeutic targets to promote physiological saliva flow, prevent salivary gland inflammation and enhance tissue regeneration.

Keywords: purinergic receptors, saliva, salivary gland dysfunction, Sjögren's syndrome, extracellular nucleotides, head and neck cancer

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***Correspondence:**
Gary A. Weisman
weismang@missouri.edu

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INTRODUCTION

Salivary gland dysfunction and the associated hyposalivation are serious clinical problems that impact millions of people (Atkinson et al., 2005; Qin et al., 2015; Siddiqui and Movsas, 2017). Saliva plays a crucial role in maintaining oral homeostasis by aiding in taste perception and digestion, protecting and lubricating oral tissues, maintaining the integrity of tooth enamel and

sustaining the oral microbiome (Dawes et al., 2015). In addition to its physiological roles, saliva contains a plethora of biomarkers and is easy to access allowing clinicians to utilize saliva as a non-invasive diagnostic material to monitor patient health (Chojnowska et al., 2018). Human saliva is increasingly being used to perform screening and risk assessment for systemic diseases, such as HIV, cancer, infections and cardiovascular disorders, demonstrating saliva's extensive clinical potential (Nunes et al., 2015). Adequate saliva production is essential for maintaining quality of life and salivary gland dysfunction leads to dry mouth, oral bacterial and yeast infections, dental caries and speech problems (Chambers et al., 2004; Meijer et al., 2009).

Hyposalivation and xerostomia (i.e., dry mouth) can present in an iatrogenic manner as side effects of over 400 medications, including antidepressants, antipsychotics, opioids, antihistamines, and others (Furness et al., 2011). Although often transient and reversible, iatrogenic xerostomia contributes to patient non-adherence to medication regimens leaving underlying pathologies untreated. Two common pathophysiological causes of salivary gland dysfunction in humans are Sjögren's syndrome (SS), an autoimmune disease characterized by xerostomia, autoantibody production and chronic lymphocytic infiltration of the salivary glands (i.e., sialadenitis), and radiotherapy-induced dysfunction where salivary glands sustain collateral damage following γ-radiation to treat head and neck tumors (Pinna et al., 2015; Mariette and Criswell, 2018). In both cases, damage to the salivary parenchyma and the failure to repair saliva-producing salivary acinar epithelium contribute to glandular dysfunction. Current therapies for salivary gland dysfunction are primarily focused on symptom management using muscarinic receptor agonists (i.e., pilocarpine or cevimeline) to stimulate saliva flow from residual salivary epithelium or through the topical use of artificial saliva (Ramos-Casals et al., 2010). While these treatments can provide some relief to patients, they are relatively ineffective because of their transient nature and failure to address the underlying inflammatory and degenerative processes that initiate and sustain glandular tissue damage. Therefore, a better understanding of the pathophysiology of salivary gland dysfunction is crucial to developing novel therapeutic approaches for this serious medical problem.

Purinergic receptors for extracellular nucleosides (i.e., adenosine) or nucleotides (i.e., ATP, ADP, UTP, UDP, and UDP-glucose) mediate numerous physiological processes, including platelet aggregation, neurotransmission, bone remodeling, and inflammatory, and immune responses (Dorsam and Kunapuli, 2004; Orriss et al., 2010; Idzko et al., 2014; Mutafova-Yambolieva and Durnin, 2014; Verkhratsky and Burnstock, 2014). In exocrine tissues, such as salivary gland, lacrimal gland and pancreas, purinergic receptor-mediated ion fluxes and cross-talk with muscarinic receptor signaling have been suggested to modulate secretory function (Novak et al., 2010; Burnstock and Novak, 2012; Hodges and Dartt, 2016). Whereas intracellular nucleotides are well-known for their role in metabolism and enzyme function, it wasn't until the 1970s that plasma membrane receptors were postulated to respond to extracellular nucleotides, including ATP and ADP, and were suggested to be responsible for non-cholinergic, non-adrenergic neurotransmission (Burnstock et al., 1972; Burnstock, 1976). Under normal conditions, extracellular nucleotides are present at minute concentrations due to the presence of ectonucleotidases (Robson et al., 2006; Zimmermann et al., 2012). However, under pathological conditions nucleotides can accumulate in the extracellular space at abnormally high concentrations, whereupon they activate local purinergic receptors in an autocrine or paracrine manner (Deaglio and Robson, 2011). The purinergic receptor family is subclassified into P1 adenosine receptors (i.e., A_1, A_{2A}, A_{2B}, and A_3) (Piirainen et al., 2011) or P2 nucleotide receptors. The P2 receptor family is further classified into metabotropic P2Y receptors (i.e., $P2Y_{1,2,4,6,11-14}$) and ionotropic P2X receptors (i.e., P2X1-7) (Abbracchio et al., 2006; Habermacher et al., 2016).

Pharmacological agonists and antagonists targeting purinergic receptors have gained widespread clinical interest and undergone clinical trials (Burnstock, 2017). P2X7 receptor (P2X7R) antagonists have been previously investigated in phase 2 clinical trials for treatment of inflammatory and autoimmune diseases, including chronic obstructive pulmonary disorder, rheumatoid arthritis and Crohn's disease (Arulkumaran et al., 2011; Keystone et al., 2012). Recent advances in the development of neuro-permeable P2X7R antagonists have stimulated interest in the use of these compounds to treat neuroinflammatory and neuropsychiatric disorders (Chrovian et al., 2014; Burnstock and Knight, 2018; Bhattacharya and Ceusters, 2019). The P2X3 receptor (P2X3R) contributes to hypersensitivity of lung afferent sensory fibers that mediate cough initiation and phase 2 clinical trials have demonstrated that the P2X3R antagonist gefapixant (AF-219) reduces refractory chronic cough in afflicted patients by 75% (Weigand et al., 2012; Abdulqawi et al., 2015). Follow-up phase 3 clinical trials are currently underway to validate the use of gefapixant for treatment of refractory chronic cough (Muccino and Green, 2019).

Due to its ability to stimulate water transport across epithelial cell membranes following activation of calcium-dependent chloride channels, the $P2Y_2$ receptor ($P2Y_2R$) agonist diquafosol has undergone human clinical trials for the treatment of dry eye disease (DED) and is currently approved for human use in Japan and South Korea under the trade name Diquas (Tauber et al., 2004; Takamura et al., 2012; Koh, 2015). A similar $P2Y_2R$ agonist, denufosol, improved lung function relative to placebo in cystic fibrosis patients during phase 2 clinical trials, but failed to achieve its primary endpoints during phase 3 follow-up trials (Accurso et al., 2011). Notably, the FDA-approved anti-coagulant Plavix (clopidogrel), a $P2Y_{12}$ receptor ($P2Y_{12}R$) antagonist, was the 2nd most prescribed drug in the world in 2010 and is currently on the World Health Organization's List of Essential Medicines (Topol and Schork, 2011; Kishore et al., 2018). However, the therapeutic potential of targeting purinergic receptors has not been well-investigated in the context of human salivary dysfunction. In the salivary glands, several purinergic receptors are expressed and upregulated under pathological conditions, including SS (Schrader et al., 2005; Baldini et al., 2013), where their activation mediates inflammatory and immune responses (Baker et al., 2008; Khalafalla M.G. et al., 2017), as well as cell repair mechanisms (El-Sayed et al., 2014). In this review, we summarize

the role of purinergic receptors in salivary gland function and highlight their potential as novel therapeutic targets to treat salivary gland dysfunction.

THE ROLE OF P2 RECEPTORS IN SALIVARY GLAND FUNCTION

The importance of saliva, as noted above, is clearly exemplified in individuals suffering from salivary gland hypofunction (Chambers et al., 2004; Atkinson et al., 2005; Meijer et al., 2009). In humans, whole unstimulated saliva is formed from the combined secretions of three pairs of major salivary glands, the submandibular (∼65%), parotid (∼20%) and sublingual (∼7%), along with numerous minor glands spread throughout the oral cavity that produce the remainder of saliva (<10%) (Humphrey and Williamson, 2001; de Almeida Pdel et al., 2008; Proctor, 2016). Upon stimulation, the parotid glands contribute the majority of total salivary secretions (Humphrey and Williamson, 2001; de Almeida Pdel et al., 2008; Proctor, 2016). Three basic cell types comprise the salivary glands: acinar epithelial cells that secrete the majority of the water and electrolytes in saliva, ductal cells that modify the electrolyte concentrations in the primary fluid and myoepithelial cells that provide contractile support for acinar cells (Martinez, 1987; Melvin et al., 2005; de Almeida Pdel et al., 2008; Proctor, 2016). Salivary acinar cells are either serous or mucous, whereas ductal cells are classified as intercalated, striated or excretory and the distribution of these cell types is dependent on species and type of gland (Melvin et al., 2005; de Almeida Pdel et al., 2008; Proctor, 2016). Along with the formation and modification of saliva, acinar and ductal cells also secrete important proteins, e.g., amylase and mucins from acinar cells (Boehlke et al., 2015; Frenkel and Ribbeck, 2015), kallikrein from ductal cells (Wong et al., 1983) and growth factors from both cell types (Masahiko et al., 2008), that are integral in maintaining the health of the oral cavity (Proctor, 2016). As shown in **Figure 1**, saliva formation is initiated in acinar cells by agonist-induced increases in intracellular Ca^{2+} levels, $[Ca^{2+}]_i$, that induce the opening of apical Ca^{2+}-dependent Cl^- channels and basolateral Ca^{2+}-dependent potassium channels, allowing Cl^- efflux into the luminal compartment and K^+ efflux into the basolateral compartment to maintain membrane potential. The negative electrochemical gradient generated by increased luminal Cl^- levels is compensated by the influx of Na^+ ions across tight junctions into the lumen leading to Na^+Cl^- accumulation followed by water movement through water channels, predominately aquaporin-5 (Ma et al., 1999), thus forming saliva in its primary isotonic form. As saliva flows through the salivary gland ducts, electrolyte modification occurs, where Na^+ and Cl^- ions are exchanged for K^+ and HCO_3^- ions by ductal cells, creating saliva in its final hypotonic form (Martinez, 1987; Melvin et al., 2005; Lee et al., 2012; Ambudkar, 2014; Proctor, 2016). Several types of Ca^{2+} mobilizing receptors are expressed on acinar cells (i.e., muscarinic, α-adrenergic, substance P), however, stimulation of the G_q protein-coupled M3 muscarinic receptor (M_3R) subtype by acetylcholine is accepted as the main receptor signaling pathway that promotes the increases in $[Ca^{2+}]_i$ necessary to enhance fluid secretion. Protein secretion from acinar and ductal cells is predominately mediated by activation of the β-adrenergic receptor (β-AR) and subsequent increases in cAMP (Melvin et al., 2005; Proctor, 2016). In addition to the canonical M_3R and β-AR pathways, a mechanism of non-cholinergic, non-adrenergic-mediated salivary flow exists (Ekström et al., 1988; Ekström, 1999; Melvin et al., 2005). Because purinergic receptor activation can result in an increase in $[Ca^{2+}]_i$ in salivary gland cells, purinergic receptor-mediated saliva production may contribute to this non-canonical pathway (Turner et al., 1998b; Melvin et al., 2005; Aure et al., 2010; Bhattacharya et al., 2015).

In other exocrine tissues, purinergic receptor signaling has been shown to modulate secretory function of acinar and ductal cells through the induction of cellular ion fluxes and cross-talk with cholinergic signaling pathways (Burnstock and Novak, 2012; Hodges and Dartt, 2016). In the pancreas, acinar cells have little functional response to exogenously applied nucleotides (Novak et al., 2002), whereas ductal cells that secrete bicarbonate and isotonic fluid express numerous functional P2X and P2Y receptors (Hede et al., 1999). In response to stimulation by acetylcholine or secretin, pancreatic ductal cell secretion is mediated by the opening of luminal Cl^- channels, including Ca^{2+}-activated Cl^- channels, as well as basolateral K^+ channels to maintain driving force for ion transport (Novak, 2008). Therefore, the finding that extracellular ATP and UTP induce increases in $[Ca^{2+}]_i$ and modulate whole cell Cl^- and K^+ conductance suggests a role for purinergic receptors in secretory regulation of pancreatic ductal cells (Christoffersen et al., 1998; Hede et al., 1999; Zsembery et al., 2000). Furthermore, studies have shown that cholinergic agonists induce ATP release from pancreatic acinar cells (Sorensen and Novak, 2001), as well as parotid and lacrimal gland cell preparations (Novak et al., 2010; Dartt and Hodges, 2011a), further supporting a role for purinergic signaling in the regulation of exocrine secretory function. In rat lacrimal gland acinar cells, extracellular nucleotide-induced protein secretion and $[Ca^{2+}]_i$ increases were inhibited by the cholinergic antagonist atropine (Dartt and Hodges, 2011a) whereas in rat parotid acinar cells extracellular nucleotides attenuated acetylcholine-induced $[Ca^{2+}]_i$ increases (Jorgensen et al., 1995; Fukushi, 1999). Although the nature of purinergic and cholinergic signaling interaction differs between exocrine tissues, these studies highlight the likely regulatory role of purinergic receptors in exocrine secretory function.

Ten years prior to the initial cloning and identification of P2 receptors, Gallacher (1982) presented the first evidence of P2 receptor activation in salivary glands. His studies demonstrated that ATP evoked a marked increase in membrane conductance, K^+ efflux and amylase secretion in the mouse parotid gland, events similar to cholinergic- and adrenergic-mediated saliva secretion (Melvin et al., 2005; Proctor, 2016). McMillian et al. (1987) showed that high extracellular ATP concentrations increased $[Ca^{2+}]_i$ in rat parotid acinar cells, the signaling response that promotes saliva production (Melvin et al., 2005).

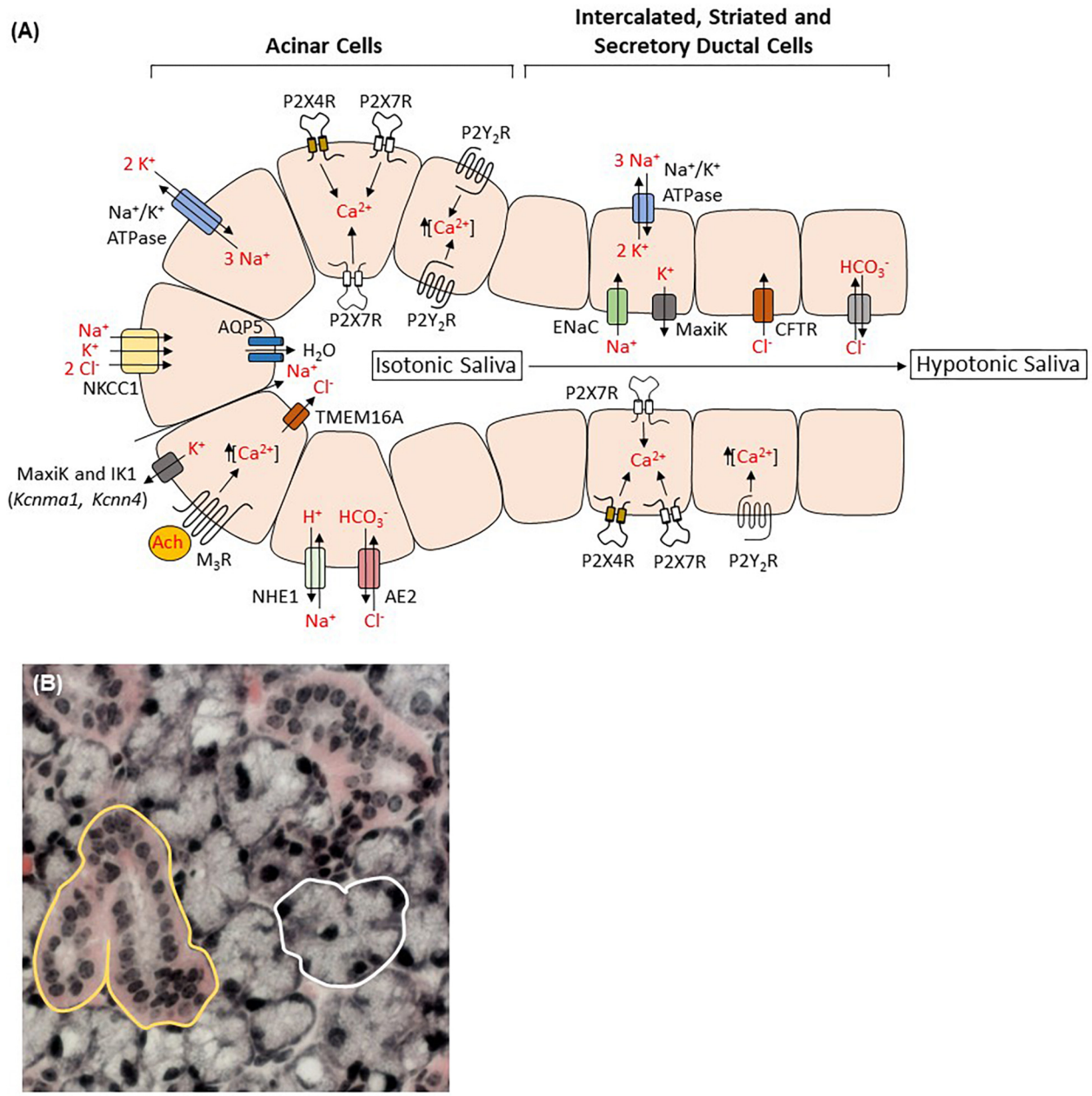

FIGURE 1 | Salivary gland acinar and ductal cells contribute to saliva formation. **(A)** Activation of type 3 muscarinic receptors (M_3R) by acetylcholine (Ach) increases release of calcium from intracellular stores and subsequent opening of the apical Ca^{2+}-dependent chloride channel transmembrane member 16A (TMEM16A; also known as anoctamin-1) and the basolateral Ca^{2+}-dependent potassium channels MaxiK (*Kcnma1*) and IK1 (*Kcnn4*), allowing Cl^- efflux into the luminal compartment and K^+ efflux into the basolateral compartment to maintain membrane potential. The combined actions of the $Na^+/K^+/2\ Cl^-$ cotransporter NKCC1, the Na^+/H^+ exchanger NHE1 and the Cl^-/HCO_3^- anion exchanger AE2 maintain the pool of intracellular Cl^- whereas the Na^+/K^+ ATPase generates the cellular Na^+ and K^+ gradients. Sodium influx down the negative electrochemical gradient into the luminal compartment is followed by water through aquaporin 5 (AQP5) water channels generating primary isotonic saliva. Modification of saliva by ductal cells involves exchanging sodium and chloride for potassium and bicarbonate through the combined actions of epithelial Na^+ channels (ENaC), cystic fibrosis transmembrane conductance regulator (CFTR) channels, MaxiK channels and perhaps Cl^-/HCO_3^- exchangers. The resulting hypotonic saliva is then secreted through ducts into the oral cavity. Functional P2X4, P2X7, $P2Y_1$, and $P2Y_2$ receptor expression has been demonstrated in both acinar and ductal cells where they may regulate secretory functions through nucleotide-induced Ca^{2+} signaling and modulation of membrane ion conductance. Available evidence suggests that P2X7 and $P2Y_2$ receptors exist on both apical and basolateral membranes while P2X4 receptors are restricted to the basolateral compartment and $P2Y_1$ receptor localization is undetermined. Importantly, P2 receptor expression in salivary gland tissue varies depending on species, isolation/culture methods and the presence of inflammatory stimuli, making definitive localization inexact. **(B)** Acinar (white) and ductal (yellow) cells outlined in a hematoxylin and eosin-stained section of a female C57BL/6 mouse submandibular gland.

Additional studies by the same group and others determined that the large ATP-induced rise in $[Ca^{2+}]_i$ was due to the influx of extracellular Ca^{2+} through a non-selective cation channel activated by the fully ionized form of ATP (i.e., ATP^{4-}) (Soltoff et al., 1990; Dehaye, 1993; McMillian et al., 1993). The order of agonist potency for channel activation in these studies was determined to be BzATP > ATP > ATPγS = 2MeSATP; thus, the receptor was classified as $P_{2Z,}$ now known as the P2X7 receptor (P2X7R) (Soltoff et al., 1990; Dehaye, 1993; McMillian et al., 1993). Thus, a physiological role for ATP in the Ca^{2+}-dependent formation of saliva was proposed, particularly since ATP was known to be released as a co-transmitter from activated sympathetic and parasympathetic nerve fibers (von Kugelgen et al., 1994; Novak, 2003). During the ensuing years, especially following the cloning, expression and identification of cDNAs for a variety of P2 receptors in the early 1990s (Lustig et al., 1993; Webb et al., 1993; Nguyen et al., 1995; Surprenant et al., 1996), several groups confirmed the expression of P2X7R in salivary gland cells and also identified and functionally characterized the ionotropic P2X4 receptor (P2X4R) and metabotropic P2Y receptors, $P2Y_1R$ and $P2Y_2R$, in these cells (Turner et al., 1999).

The P2X7R is a 595 amino acid protein that includes two transmembrane domains, intracellular carboxy and amino termini and a bulky hydrophilic extracellular loop with a cysteine rich region that forms disulfide bridges (McCarthy et al., 2019). It shares 40–50% amino acid homology with the other P2X receptors, but is structurally distinct in that its C-terminal tail extends for an additional 100–200 amino acids (North, 2002; Adinolfi et al., 2005; Sluyter, 2017). The P2X7R is activated by high extracellular ATP (eATP) concentrations (>100 μM) with brief stimulation (10–30 s) causing the depolarization of the plasma membrane due to the opening of a membrane cation channel that promotes the influx of Na^+ and Ca^{2+} and the efflux of K^+ (Weisman et al., 1984, 1989; Adinolfi et al., 2005). Sustained P2X7R activation induces the opening of a pore permeable to hydrophilic molecules up to 900 Da, and promotes production of reactive oxygen species (ROS), NLRP3 inflammasome-dependent IL-1β release, extensive plasma membrane blebbing and ultimately cell death (Weisman et al., 1984, 1989; Woods et al., 2012; Di Virgilio et al., 2017; Giuliani et al., 2017; Khalafalla M.G. et al., 2017). The P2X7R is widely expressed in diverse tissues, including hematopoietic cells (Feng et al., 2016), neurons (Miras-Portugal et al., 2017), glia (Stokes et al., 2015; Kaczmarek-Hajek et al., 2018), bone (Agrawal and Gartland, 2015), muscle (Fabbrizio et al., 2019), endothelium (Green et al., 2018), epithelium (Woods et al., 2012), and immune cells (Ferrari et al., 1997). In the exocrine pancreas, P2X7Rs have been shown to be primarily expressed in pancreatic ductal cells where they may contribute to secretory regulation through induction of cation fluxes and interaction with cholinergic signaling (Novak et al., 2010; Burnstock and Novak, 2012). Similarly, in lacrimal glands P2X7Rs mediate $[Ca^{2+}]_i$ increases, ERK1/2 activation, protein secretion and modulate both cholinergic and adrenergic receptor signaling pathways (Hodges et al., 2009; Dartt and Hodges, 2011a,b). After its initial characterization in rat parotid acinar cells (McMillian et al., 1987; Gibbons et al., 2001), P2X7R expression and function were reported to promote increases in $[Ca^{2+}]_i$ in rat submandibular acinar cells (Lee et al., 1997; Alzola et al., 2001), murine parotid (Li et al., 2003; Reyes et al., 2008; Bhattacharya et al., 2012) and submandibular acinar cells (Nakamoto et al., 2009) and human parotid acinar cells (Brown et al., 2004).

In addition to numerous studies defining its role in mediating inflammatory and immune responses in disease models (Savio et al., 2018; Cao et al., 2019; Zeng et al., 2019), including those pertaining to salivary glands (Woods et al., 2012; Khalafalla M.G. et al., 2017), there is evidence that P2X7Rs regulate salivary secretory function (Nakamoto et al., 2009; Novak et al., 2010; Pochet et al., 2013). Along with its ability to increase $[Ca^{2+}]_i$ due to calcium influx, P2X7R activation has been shown to inhibit mobilization of intracellular Ca^{2+} induced by muscarinic or substance P receptor agonists in rat submandibular acinar cells (Hurley et al., 1993; Metioui et al., 1996) and cholinergic mobilization of $[Ca^{2+}]_i$ was significantly increased in parotid acinar cells prepared from P2X7R-null ($P2X7R^{-/-}$) mice (Novak et al., 2010). The mechanism of this inhibition is still unclear, but it does not appear to be due to interference with binding of the autonomic agonists to their receptors (Hurley et al., 1993). This observation was corroborated in an *ex vivo* murine submandibular gland (SMG) preparation, where co-stimulation with ATP and muscarinic receptor agonists had an inhibitory effect on the gland's saliva production (Nakamoto et al., 2009). Further, in glands prepared from $P2X7R^{-/-}$ mice the inhibitory effect of ATP on carbachol-induced saliva secretion was abolished, suggesting an inhibitory role for P2X7Rs in saliva production (Nakamoto et al., 2009). However, in this same study ATP or BzATP alone evoked fluid secretion in a time-dependent manner that was greatly reduced in glands from $P2X7R^{-/-}$ mice, whereas carbachol alone induced similar saliva secretion in wild type and $P2X7R^{-/-}$ glands. Similarly, another study found no significant difference in cholinergic-mediated whole saliva secretion in $P2X7R^{-/-}$ mice compared to wild type (Pochet et al., 2007). In contrast, Novak et al. (2010) found that cholinergic-mediated whole saliva secretion was significantly decreased in $P2X7R^{-/-}$ mice, as compared to wild type mice, and this was particularly evident in male mice. While the reasons for the disparities among these studies are unclear, they may be due to differences in the type of saliva collected (i.e., whole saliva vs. saliva from specific glands), methods of induction of saliva secretion, tissue specificity, sex, or mouse strain.

The P2X7R is also expressed in rat (Lee et al., 1997; Alzola et al., 1998) and mouse salivary ductal cells (Li et al., 2003; Pochet et al., 2007; Nakamoto et al., 2009), suggesting participation in the modification of the electrolyte content of saliva. Studies indicate no difference in $[Na^+]$ or $[Cl^-]$ in muscarinic agonist-induced whole saliva secreted in wild type compared to $P2X7R^{-/-}$ mice, however the $[K^+]$ was elevated in $P2X7R^{-/-}$ mouse whole saliva (Pochet et al., 2007). Since the majority of the K^+ in saliva originates from ductal cells, it has been hypothesized that ATP released from acinar cells during exocytosis stimulates ductal P2X7Rs that regulate the activity of K^+ channels located on the apical membrane (Liu et al., 1999; Bhattacharya et al., 2015). In addition to K^+ modification, activation of P2X7Rs in

ductal cells increases phospholipase A2-dependent secretion of arachidonic acid, a precursor of prostaglandin E2 (PGE_2), and kallikrein (Alzola et al., 1998) into saliva (Pantano et al., 2019). Interestingly, cell lines of salivary origin exhibit low expression and function of P2X7R, which are enhanced following DNA demethylation (Shin et al., 2015).

Another P2X ionotropic receptor expressed in salivary acinar and ductal cells is the P2X4R (Turner et al., 1998b). Unlike the P2X7R's requirement for activation by high eATP concentrations, P2X4Rs have nanomolar affinity for ATP (North, 2016; Suurvali et al., 2017) and were initially found to regulate the biphasic response to ATP in rat parotid gland cells (McMillian et al., 1993). The P2X4R is widely expressed in a variety of cell types, e.g., neurons and microglia (Ho et al., 2014), epithelium (Casas-Pruneda et al., 2009), and endothelium (Lv et al., 2015), and P2X4R expression in microglia is notable for the key role it plays in mediating neuropathic pain (Inoue, 2019). Although RT-PCR analysis has identified P2X4R expression in pancreatic acinar and ductal cells (Luo et al., 1999; Novak et al., 2002) and lacrimal gland acinar cells (Hodges et al., 2011; Kamada et al., 2012), its functional role in exocrine tissues remains largely unexplored. Physical interactions between P2X4Rs and P2X7Rs have been demonstrated, although the nature of this interaction remains controversial (Kopp et al., 2019). Some studies suggest that P2X4R and P2X7R subunits form heteromeric channels (Guo et al., 2007; Schneider et al., 2017), while others conclude that P2X4 and P2X7 receptors interact in their respective homotrimeric form (Nicke, 2008; Boumechache et al., 2009; Antonio et al., 2011). Furthermore, P2X4R expression has been localized to lysosomal membranes, whereas P2X7Rs primarily reside at the plasma membrane (Guo et al., 2007; Huang et al., 2014). Nevertheless, studies have also demonstrated functional evidence for P2X4R/P2X7R interactions (Guo et al., 2007; Kawano et al., 2012; Perez-Flores et al., 2015). In salivary epithelium, P2X4Rs modulate P2X7R-mediated ion flow and ethidium bromide dye uptake (Casas-Pruneda et al., 2009), suggesting a functional interaction that regulates physiological processes, including plasma membrane ion channel function and pore formation. Importantly, the interaction between these two purinergic receptors results in a decreased sensitivity to ATP, as compared to the P2X4R or P2X7R alone, suggesting the formation of heteromeric channels with novel functional and pharmacological properties (Casas-Pruneda et al., 2009).

While the contribution of P2X4R activation to physiological saliva production has not been explored, *ex vivo* murine SMG preparations from $P2X7R^{-/-}$ mice exhibit weak ATP-induced saliva secretion that could be attributed to P2X4R activation (Nakamoto et al., 2009). As seen previously with muscarinic or adrenergic receptor activation (Baldys-Waligorska et al., 1987; Yoshimura and Hiramatsu, 1998; Tanimura et al., 1999; Bruce et al., 2002), co-stimulation of β-adrenergic receptors and P2X7Rs or P2X4Rs enhanced the influx of Ca^{2+} in mouse parotid acinar cells, as compared to activation of either receptor alone (Bhattacharya et al., 2015). In contrast, studies using human parotid acinar cells found this co-stimulatory effect only between the P2X4R and β-adrenergic receptor (Brown et al., 2004). Taken together, the expression of both P2X7Rs and P2X4Rs in salivary glands supports the idea that they are involved in the interplay between canonical and non-canonical signaling pathways that regulate saliva flow and composition and their involvement is likely dependent on their tissue localization (i.e., basal vs. apical) in polarized acinar and/or ductal epithelial cells (Bhattacharya et al., 2012, 2015).

The metabotropic $P2Y_1$ receptor ($P2Y_1R$), formerly known as the P_{2T} receptor, has been identified and cloned (Webb et al., 1993; Baranska et al., 2017) and has features typical of G protein-coupled receptors, i.e., an extracellular N-terminus and an intracellular C-terminus, seven hydrophobic transmembrane regions, three extracellular loops and three intracellular loops (von Kugelgen and Hoffmann, 2016). The $P2Y_1R$ has a distinctive rank order of agonist potencies (i.e., 2-methylthio-ADP > ADP > ATP) and its activation induces canonical $G\alpha_q$ signaling leading to phospholipase C activation and generation of the second messengers inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol that increase $[Ca^{2+}]_i$ and protein kinase C (PKC) activity, respectively (von Kugelgen and Wetter, 2000; Abbracchio et al., 2006; Baranska et al., 2017; von Kugelgen, 2019). Additionally, $P2Y_1R$ activation stimulates metalloprotease-dependent transactivation of the epidermal growth factor receptor (EGFR) (Buvinic et al., 2007) and mitogen-activated protein kinase (MAPK) activity through activation of phosphatidylinositol 3-kinase, Src kinase and PKC (Sellers et al., 2001; Baranska et al., 2017). The $P2Y_1R$ is widely distributed in mammalian tissues and is involved in many physiological and biochemical responses, such as platelet aggregation (Fabre et al., 1999), pain sensation (Barragan-Iglesias et al., 2015), vasodilation (Zerr et al., 2011), bone remodeling (Orriss et al., 2011), and osmotic volume regulation (Grosche et al., 2013). In exocrine tissues, immunofluorescence and RT-PCR analyses provide evidence of $P2Y_1R$ expression in pancreatic ductal cells where $P2Y_1R$ agonists also induce $[Ca^{2+}]_i$ increases (Luo et al., 1999; Coutinho-Silva et al., 2001). However, the role of $P2Y_1Rs$ in exocrine pancreas function has been unexplored. Likewise, $P2Y_1R$ expression has been demonstrated in lacrimal acinar cells and myoepithelial cells by RT-PCR, immunofluorescence and measurement of $P2Y_1R$ agonist-induced $[Ca^{2+}]_i$ increases, but further functional analyses are lacking (Ohtomo et al., 2011). Interestingly, the $P2Y_1R$ has been used as a surrogate cell-surface marker for the nuclear protein pancreatic duodenal homeobox 1 (PDX1) to isolate progenitor-like ductal cells from human pancreatic tissues, although no functional role for $P2Y_1Rs$ was investigated (Qadir et al., 2018). In contrast, studies on endocrine pancreas function suggest a role for $P2Y_1Rs$ in mediating insulin secretion from β cells (Leon et al., 2005; Petit et al., 2009). The $P2Y_1R$ is also involved in tissue development, as was first described in chick embryos (Meyer et al., 1999; Meyer et al., 2001) and more recently in the developing brain (Huang et al., 2019). In the developing rat salivary gland, it was observed that acinar cells prepared from immature glands of 1 day-old pups had a robust $[Ca^{2+}]_i$ response to $P2Y_1R$ agonists, whereas acini prepared from adult rat salivary glands had no response (Park et al., 1997). Interestingly, $P2Y_1R$ mRNA expression remained the same at all ages in rats, suggesting that the loss of the $P2Y_1R$-mediated $[Ca^{2+}]_i$

response may be due to age-dependent alterations in intracellular G protein coupling (Park et al., 1997). A subsequent study using rat SMG acinar and ductal cell preparations confirmed the age-dependent reduction in $P2Y_1R$-mediated increases in $[Ca^{2+}]_i$ and, similarly, found unchanged $P2Y_1R$ expression levels at all ages (Baker et al., 2006). This study further demonstrated that $P2Y_1R$-mediated activation of the MAPKs, extracellular signal-regulated kinases 1 and 2 (ERK1/2), was consistent in rats of all ages, indicating that ERK1/2 activation is independent of $P2Y_1R$-mediated changes in $[Ca^{2+}]_i$. Western analysis and assays of GTPγ^{35}S binding to G proteins determined that the age-dependent decrease in $P2Y_1R$ activity in rat SMG cells was due to both decreased expression of the 52 kDa $G\alpha_{14}$ protein and differential coupling of $P2Y_1Rs$ to $G\alpha_{q/11}$ with age (Baker et al., 2006). These studies suggest that $P2Y_1Rs$ use diverse mechanisms for coupling to multiple G proteins that regulate a variety of physiological responses during development. To date, these findings have not been confirmed in salivary glands of mice, but with the availability of $P2Y_1R$-null mice, it would be of interest to assess the role of this receptor in salivary gland morphology and function during development.

The $P2Y_2R$ (formerly known as the P_{2U} receptor), equipotently activated by ATP or UTP ($EC_{50} \sim 2\ \mu M$), is the only other known $G\alpha_q$-coupled purinergic receptor identified in salivary glands (Turner et al., 1998b, 1999) and has been cloned and functionally characterized in mice and humans (Erb et al., 1993; Lustig et al., 1993; Parr et al., 1994). Similar to the $P2Y_1R$, $P2Y_2R$ activation induces canonical $G\alpha_q$ signaling leading to increases in $[Ca^{2+}]_i$ and PKC activation, and the $P2Y_2R$ is expressed in numerous cell and tissue types, e.g., neurons (Peterson et al., 2013), epithelium (Shishikura et al., 2016; Wu et al., 2017), endothelium (Seye et al., 2003) and immune cells (Idzko et al., 2014; Woods et al., 2018), where it modulates a variety of cellular responses, including neurotransmission (Zhang and Li, 2019), proliferation (Shen et al., 2004), cell migration (Bagchi et al., 2005), cytoskeletal rearrangements (Liao et al., 2007), and ion fluxes (Murakami et al., 2004). The diversity of cellular responses mediated by $P2Y_2Rs$ is due, in part, to unique structural features enabling activation of multiple signal transduction pathways. In addition to canonical $G\alpha_q$ signaling (Parr et al., 1994), the $P2Y_2R$ contains a motif typically found in extracellular matrix proteins, i.e., an Arg-Gly-Asp (RGD)-sequence, in its first extracellular loop that binds to $\alpha_v\beta_3/\beta_5$ integrins to activate G_o and G_{12} proteins, enhance MAPK (ERK1/2) phosphorylation and regulate ATP- and UTP-induced cell chemokinesis and chemotaxis (Erb et al., 2001; Bagchi et al., 2005; Wang et al., 2005; Liao et al., 2007). Within the intracellular C-terminus of the $P2Y_2R$, Src-homology-3 (SH3) binding domains (PXXP) enable the $P2Y_2R$ to bind and activate the tyrosine kinase Src, enabling nucleotide-induced, Src-dependent transactivation of growth factor receptors and downstream MAPKs that regulate cell proliferation and migration (Liu et al., 2004; Seye et al., 2004). Additionally, interaction of the $P2Y_2R$ C-terminus with the actin-binding protein filamin-A contributes to cell migration and Rho GTPase-mediated cytokine release (Yu et al., 2008; Seye et al., 2012). The $P2Y_2R$ also mediates the proprotein convertase furin-dependent activation of metalloproteases, i.e., a disintegrin and metalloproteinase 10 and 17 (ADAM10/17), to cleave transmembrane proteins (Camden et al., 2005), thereby releasing EGFR/ERB ligands that promote Src-independent EGFR activation (Ratchford et al., 2010). These diverse $P2Y_2R$ signaling pathways have been implicated in a number of pathologies, including Alzheimer's disease (Ajit et al., 2014), cardiovascular disease (Chen et al., 2017), cancer (Hu et al., 2019), SS (Woods et al., 2018), and hantavirus cardiopulmonary syndrome (Bondu et al., 2018), as well as processes such as wound healing (Jin et al., 2014) and tissue regeneration (El-Sayed et al., 2014).

In exocrine tissues such as the lacrimal gland, RT-PCR and immunohistochemical analyses have identified $P2Y_2R$ expression in acinar and ductal cells (Kamada et al., 2012; Tanioka et al., 2014). While no functional response to the $P2Y_2R$ agonist UTP was observed in lacrimal acinar cells (Kamada et al., 2012), cultured lacrimal gland myoepithelial cells do exhibit increased $[Ca^{2+}]_i$ in response to extracellular UTP suggesting the presence of $P2Y_2$ or $P2Y_4$ receptors (Ohtomo et al., 2011). In the exocrine pancreas, RT-PCR and immunohistochemical analyses indicate that $P2Y_2Rs$ are expressed in both pancreatic acini (Novak et al., 2002) and ductal cells (Hede et al., 1999; Luo et al., 1999; Coutinho-Silva et al., 2001), although very few pancreatic acinar cells show functional responses to extracellular ATP or UTP (Novak et al., 2002). In pancreatic ductal cells, $P2Y_2R$-mediated increases in $[Ca^{2+}]_i$ altered whole-cell K^+ conductance (Hede et al., 1999), likely through modulation of Ca^{2+}-activated K^+ channels (Hede et al., 2005), suggesting a role in the regulation of ductal fluid flow and Cl^-/HCO_3^- levels. Studies with pancreatic ductal cell lines have also shown that the $P2Y_2R$ agonists ATP and UTP increase membrane Cl^- conductance through the opening of Ca^{2+}-dependent Cl^- channels (Galietta et al., 1994; Chan et al., 1996; Zsembery et al., 2000). The ability of $P2Y_2Rs$ to induce chloride secretion and subsequent fluid flow across epithelial cell membranes led to investigation of the $P2Y_2R$ as a therapeutic target for cystic fibrosis (Weisman et al., 1998; Kellerman et al., 2002; Lazarowski and Boucher, 2009). By stimulating Ca^{2+}-dependent Cl^- secretion, topical application of the selective $P2Y_2R$ agonist diquafosol has been shown to promote tear secretion and is currently being used to treat DED (Jacobson and Civan, 2016).

In 1991, the $P2Y_2R$ was first identified in a cell line of salivary gland origin, human salivary gland (HSG) cells, where it was shown to mediate UTP-induced IP_3 production and increases in $[Ca^{2+}]_i$ and plasma membrane K^+ transport (Yu and Turner, 1991). A subsequent study determined that exposure of HSG cells to UTP potentiated a regulatory volume decrease (RVD) after hypotonic stress, suggesting that activation of $P2Y_2Rs$ provides the driving force for net Cl^- efflux that enables the cells to rapidly restore their volume (Kim et al., 1996), a response that occurs during salivary secretion (Melvin et al., 2005). In 1998, it was shown that simian virus 40-transformed salivary cell lines from rat SMG and parotid glands (Quissell et al., 1998), unlike HSG cells, were suitable for Ussing chamber studies due to their ability to form polarized cell monolayers (Turner et al., 1998a). Using the polarized rat parotid cell line Par-C10 in a Ussing chamber, transepithelial resistance measurements determined

that functional $P2Y_2R$ expression was localized to the apical membrane, consistent with its localization in other epithelium (Hwang et al., 1996; Chan et al., 1997; Yang et al., 2009), and its activation by UTP increased an anion (Cl^-/HCO_3^-)-dependent change in short-circuit current (I_{sc}) (Chan et al., 1996, 1997; Clarke et al., 1999). Taken together, these results suggest that expression of $P2Y_2Rs$ on salivary gland epithelium may contribute to saliva secretion; however, subsequent studies with freshly isolated salivary acinar cells showed little evidence of $P2Y_2R$ expression or activity under steady-state conditions (Turner et al., 1997; Ahn et al., 2000; Schrader et al., 2005). Moreover, carbachol-stimulated whole saliva secretion in $P2Y_2R$-null mice ($P2Y_2R^{-/-}$) is unchanged compared to wild type mice (Woods et al., 2018), suggesting that $P2Y_2Rs$ do not contribute to overall fluid secretion. Earlier studies demonstrated UTP-induced Cl^- fluxes in rat salivary duct cells (Lee et al., 1997; Zeng et al., 1997) with one study suggesting that $P2Y_2R$ expression on striated ducts regulates CFTR activity (Ishibashi et al., 2008), thereby possibly modifying the ionic content of saliva.

THE ROLE OF P2 RECEPTORS IN SALIVARY GLAND INFLAMMATION

The contribution of P2 receptors to physiological salivary gland function is predicated on the presence of endogenous agonists (i.e., extracellular nucleotides) in sufficient concentrations to activate their cognate receptors, as is the case when ATP is co-released with neurotransmitters from sympathetic and parasympathetic nerves (von Kugelgen et al., 1994; Novak, 2003). In exocrine tissues such as the pancreas and lacrimal glands, ATP is released in response to stimulation by physiological agonists such as acetylcholine and cholecystokinin-8 (Sorensen and Novak, 2001; Yegutkin et al., 2006; Novak et al., 2010; Dartt and Hodges, 2011a). Additionally, measurable amounts of ATP are present in rat saliva induced by intraperitoneal pilocarpine administration (Ishibashi et al., 2008). However, the concentration of extracellular nucleotides is tightly regulated under physiological conditions and maintained in the low μM range by ectonucleotidases (Pellegatti et al., 2008; Di Virgilio et al., 2018), such as the nucleoside triphosphate diphosphohydrolase ENTPD1 (CD39) and related family members (Deaglio and Robson, 2011; Zimmermann et al., 2012). Using conventional luciferin/luciferase luminescence measurements or cell-based biosensors, the concentration of extracellular ATP released from pancreatic acinar or β cells has been measured at ∼10–25 μM (Hazama et al., 1998; Sorensen and Novak, 2001), although *in vivo* measurement of absolute extracellular nucleotide concentrations is an active area of research (De Marchi et al., 2020). However, during periods of inflammation or other cellular stresses, such as hypoxia in the tumor microenvironment, extracellular ATP levels have been shown to exceed 100 μM and are likely much higher in the context of the confined pericellular space (Pellegatti et al., 2008; Joo et al., 2014; Di Virgilio et al., 2018; De Marchi et al., 2019). Immune and apoptotic cells release ATP through connexin and pannexin hemichannels during inflammatory responses and uncontrolled release of intracellular ATP pools can also occur during cell necrosis (Eltzschig et al., 2006; Chekeni et al., 2010). Mounting evidence also suggests that connexin 43-mediated ATP release from γ-irradiated cells causes the radiation-induced bystander effect where adjacent, non-irradiated cells exhibit physiological responses mediated by P2 receptors (Tsukimoto et al., 2010; Ohshima et al., 2012; Tsukimoto, 2015; Kojima et al., 2017). Interestingly, the ionotropic P2X7 receptor also has been shown to mediate ATP release (Suadicani et al., 2006; Ohshima et al., 2010), likely through its sustained activation that leads to membrane depolarization and pore formation (Dahlquist et al., 1974; Weisman et al., 1984; Buisman et al., 1988), and P2X7R blockade has been shown to attenuate ionizing radiation (IR)-induced ATP release from salivary acinar cells (Gilman et al., 2019). Recognizing that salivary gland inflammation and radiation exposure, two common sources of salivary gland dysfunction, promote the release of extracellular nucleotides and subsequent P2 receptor activation, defining the role of P2 receptors in salivary gland pathophysiology has been an area of intense interest.

In addition to its role as an ion channel, activation of the P2X7R initiates signaling cascades that produce pro-inflammatory cytokines (e.g., IL-1β, IL-18, IL-6, IL-8, and TNF-α) to enable antigen-presenting cells to initiate innate immune responses (Ferrari et al., 1997; Solini et al., 1999; Mehta et al., 2001; Lister et al., 2007; Shieh et al., 2014). In salivary epithelium, our group has shown that P2X7R activation with ATP or BzATP triggers apoptotic and pro-inflammatory cell responses, including increases in caspase-1 and caspase-3 activity and immune cell infiltration into wild type, but not $P2X7R^{-/-}$, mouse SMGs (Woods et al., 2012). Also, P2X7R activation in salivary epithelium was found to induce the assembly of the NLRP3 inflammasome multiprotein complex and the subsequent release of IL-1β, a response that was dependent on K^+ efflux, production of ROS and functional heat shock protein 90 (Khalafalla M.G. et al., 2017). P2X7R activation also has been shown to mediate the protease-dependent release of α-fodrin (Woods et al., 2012), a putative autoantigen associated with SS (Miyazaki et al., 2005), through a mechanism that requires caspase-3 and calpain enzymatic activities (Hwang et al., 2009b). P2X7R activation induces membrane blebbing, an early indicator of cell apoptosis, in salivary epithelial cells isolated from wild type, but not $P2X7R^{-/-}$, mice (Woods et al., 2012). The mechanism of P2X7R-mediated membrane blebbing was shown to require sustained elevation of $[Ca^{2+}]_i$, activation of the ROCK I signaling pathway and phosphorylation of myosin light chain, but does not involve caspase-3 activation (Hwang et al., 2009a).

There are increasing lines of evidence that P2X7R-induced pro-inflammatory responses are modulated by the P2X4R as well. In immune cells, P2X4Rs have been shown to modulate P2X7R-induced IL-1β release and dye uptake through interaction with the P2X7R C-terminus and P2X4R antagonism abolished P2X7R-induced Ca^{2+} influx and IL-1β and IL-18 release (Sakaki et al., 2013). In gingival epithelial cells, P2X7Rs, P2X4Rs and pannexin-1 hemichannels were all required for ATP-induced ROS production, NLRP3 inflammasome activation and IL-1β release (Hung et al., 2013). These cellular mechanisms may also

be important in IL-1β release from salivary epithelium, where P2X4Rs have been shown to modulate P2X7R-mediated ion flow and pore formation (Casas-Pruneda et al., 2009).

In rodent salivary glands, $P2Y_2R$ expression is negligible under physiological conditions. Interestingly, freshly dispersed salivary epithelial cells significantly upregulated $P2Y_2R$ expression and activity as a function of time when placed in culture (Turner et al., 1997; El-Sayed et al., 2014), consistent with a possible role for $P2Y_2R$ in the cellular response to stress. $P2Y_2R$ upregulation also occurs in the *in vivo* ductal ligation model of salivary gland inflammation and fibrosis (Ahn et al., 2000) and has been similarly seen in other *in vivo* models of stress and inflammation, i.e., intestinal inflammation (Grbic et al., 2008), rat vascular neointima formation after balloon angioplasty (Seye et al., 1997), collared rabbit carotid arteries (Seye et al., 2002), glomerulonephritis (Rennert et al., 2018), myocardium of rats with congestive heart failure (Granado et al., 2015) and mouse models of the autoimmune disease SS (Schrader et al., 2005; Woods et al., 2018). IL-1β has been previously shown to induce $P2Y_2R$ upregulation (Kong et al., 2009; Peterson et al., 2013), likely through binding of NF-κB p65 to the *$P2Y_2R$* promoter region that has been demonstrated to mediate inflammation-induced $P2Y_2R$ upregulation in human intestinal epithelial cells (Degagne et al., 2009). Taken together, these studies suggest that ATP released from stressed cells during inflammation activates P2X7Rs to induce the release of IL-1β and other cytokines. Subsequent activation of IL-1 receptors by IL-1β in surrounding cells induces $P2Y_2R$ upregulation and further downstream responses to ATP and UTP. In this way, the release of a single alarmin (e.g., ATP or UTP) in response to cellular stress can locally modulate a wide range of signaling pathways to fine-tune the tissue response to inflammatory stimuli.

In HSG cells, UTP-induced activation of $P2Y_2Rs$ has been shown to regulate localized immune responses and the binding of immune cells through the upregulation of the cell adhesion molecule VCAM-1 via an EGFR-dependent mechanism (Baker et al., 2008). Furthermore, $P2Y_2R$ activation has been shown to stimulate the production and secretion of pro-inflammatory lymphotoxin-α (LT-α), a member of the tumor necrosis factor family of cytokines that is required for the development of lymphoid tissues and mediates interactions between immune cells (Shen et al., 2010, 2013), suggesting multiple mechanisms whereby $P2Y_2Rs$ regulate localized immune responses relevant to salivary gland inflammation (Seye et al., 2012; Qian et al., 2016; Woods et al., 2018).

P2 RECEPTORS IN SJÖGREN'S SYNDROME

A number of autoimmune inflammatory diseases are reported to impact the function of salivary glands, including rheumatoid arthritis (Nagler et al., 2003; Helenius et al., 2005; Zalewska et al., 2011), systemic lupus erythematosus (SLE) (Leite et al., 2015) and diabetes mellitus (Moore et al., 2001). One of the major causes of salivary gland dysfunction is chronic inflammation associated with the autoimmune disease SS, the 2nd most common autoimmune rheumatic disease in the U.S., in which unresolved inflammation of the salivary and lacrimal glands contributes to tissue degeneration and subsequent loss of function (Helmick et al., 2008; Vivino, 2017). Clinical classification criteria for primary SS (pSS) in the absence of other autoimmune diseases include the presence in blood serum of anti-Ro/SSA and anti-La/SSB autoantibodies to their intracellular antigens, increased corneal staining using fluorescein dye (ocular staining score ≥ 5), decreased tear (Schirmer's test $\leq$ 1 mm/min) and saliva ($\leq$ 0.1 ml/min) flow rates and the presence of focal lymphocytic sialadenitis (focus score $\geq$ 1 foci/4 mm^2) in minor salivary gland biopsies (Shiboski et al., 2017). During SS pathogenesis, T and B cells (van Woerkom et al., 2005; Daridon et al., 2006), dendritic cells (Ozaki et al., 2010; Zhao et al., 2016), and macrophages (Manoussakis et al., 2007) accumulate in the salivary glands where, along with salivary gland epithelial cells, they produce numerous pro-inflammatory cytokines, including IFN-γ, B cell-activating factor, TNF-α, IL-1β, IL-6 and IL-18, which initiate pro-inflammatory immune responses that ultimately degenerate the salivary glands (Hulkkonen et al., 2001; Willeke et al., 2003; Daridon et al., 2007; Sakai et al., 2008; Nezos et al., 2015). Additionally, SS patients produce high levels of immunoglobulins and autoantibodies besides anti-Ro/SSA and anti-La/SSB (Nardi et al., 2006; Suresh et al., 2015), including anti-α-fodrin (Watanabe et al., 1999; Miyazaki et al., 2005), RF (rheumatoid factor) (Müller et al., 1989; Huo et al., 2010) and other autoantibodies (Ramos-Casals et al., 2006; Shen et al., 2014; Suresh et al., 2015) that have been previously reported to activate intrinsic and extrinsic apoptotic pathways in salivary gland cells (Sisto et al., 2006; Lisi et al., 2007). Furthermore, anti-muscarinic receptor-3 autoantibodies that inhibit saliva production and aquaporin translocation to the plasma membrane (Bacman et al., 1996; Dawson et al., 2006) have been identified in the blood serum of SS patients. Taken together, these data suggest that chronic auto-inflammatory responses along with autoantibody-induced reductions in saliva and tear production and increased salivary acinar cell apoptosis contribute to pSS pathogenesis that ultimately leads to salivary gland dysfunction and fibrosis as well as systemic pathologies (i.e., chronic fatigue, lymphoma development, and secondary autoimmune manifestations).

Previous studies have demonstrated that the expression of *P2X7R*, *caspase-1*, *IL-1β*, *IL-18* and components of the NLRP3 inflammasome multiprotein complex are significantly increased in labial salivary gland biopsies from SS patients, which positively correlates with salivary gland focus score (# of mononuclear cell foci/4 mm^2 tissue area) (Baldini et al., 2013, 2017). Furthermore, these studies found that when SS patients were stratified based on the presence of anti-Ro/SSA autoantibodies, the increased expression of *P2X7R* and NLRP3 inflammasome components was even more pronounced in seropositive cohorts compared to seronegative cohorts (Baldini et al., 2013, 2017). Subsequent immunofluorescence analysis indicated that P2X7R expression in SS salivary gland biopsies co-localized with the acinar epithelial cell marker aquaporin 5, rather than immune cell markers, suggesting that P2X7Rs on salivary gland epithelium contribute to SS pathogenesis through a process termed autoimmune epithelitis (Mitsias et al., 2006; Baldini et al., 2017). Additionally,

this prospective study of 147 SS patients over ∼5 years found that those who eventually developed mucosa-associated lymphoid tissue non-Hodgkin lymphoma (MALT NHL), a serious complication of SS, had significantly higher labial salivary gland *P2X7R* expression at the time of SS diagnosis compared to non-lymphoma SS patients, suggesting that P2X7R expression may be a useful biomarker for MALT NHL development (Baldini et al., 2017). In an analysis of *P2X7R* functional polymorphisms in 114 SS patients and 136 non-SS controls, the frequency of a single nucleotide polymorphism in exon 13 (A1405G, rs2230912) was significantly increased in seropositive SS patients, as compared to control subjects (Lester et al., 2013). As determined by ATP-induced ethidium bromide uptake to detect P2X7R activation in isolated peripheral blood lymphocytes, the P2X7R A1405G polymorphism was found to be a gain-of-function mutation that was suggested to be a risk factor for seropositive SS in the absence of other SS-associated human leukocyte antigen risk alleles. However, this A1405G association failed to be replicated in a larger patient cohort (Lester et al., 2013).

Antagonism of the P2X7R, whose encoding gene is located within a mapped SLE susceptibility region on chromosome 12 (Elliott et al., 2005), has been investigated as a potential treatment for several inflammatory diseases, including SLE (Turner et al., 2007; Taylor et al., 2009), rheumatoid arthritis (Arulkumaran et al., 2011) and chronic obstructive pulmonary disease (Lucattelli et al., 2011). Due to its increased expression in salivary gland biopsies from SS patients (Baldini et al., 2013) and its reported role in the activation of pro-inflammatory responses in salivary epithelium (Woods et al., 2012), the P2X7R has emerged as an appealing therapeutic target to treat SS. Our group reported that *in vivo* inhibition of P2X7Rs using the competitive antagonist A-438079 significantly reduced sialadenitis and improved carbachol-induced saliva flow in the NOD.H-2^{h4}, $CD28^{-/-}$, $IFN\gamma^{-/-}$ murine model of SS-like salivary gland autoimmune exocrinopathy (Khalafalla M.G. et al., 2017). P2X7R antagonism also significantly reduced salivary gland expression of immunoactive molecules known to be upregulated in salivary gland biopsies isolated from SS patients, including IL-1β, ICAM, VCAM, E-selectin, CD80, and CD86 (Tsunawaki et al., 2002; Khalafalla M.G. et al., 2017). Taken together, these studies suggest that the P2X7R represents a promising target for therapeutic intervention in salivary gland inflammation.

Previous studies have demonstrated that the $P2Y_2R$ is upregulated in major salivary glands of several mouse models of SS, including NOD.B10 (Schrader et al., 2005), IL-14α transgenic (IL-14αTG) (Woods et al., 2018) and C57BL/6-NOD.*Aec1Aec2* mice (unpublished observations). It was recently reported by our group that $P2Y_2R$ expression was increased in both SMG epithelium and SMG-infiltrating B cells in aged IL-14αTG mice with SS-like disease and genetic deletion of the $P2Y_2R$ attenuated both B and T cell infiltration of the salivary glands (Woods et al., 2018). Additionally, attenuated sialadenitis following $P2Y_2R$ deletion correlated with significantly reduced levels of LT-α in salivary gland epithelial cells and infiltrating immune cells, suggesting that $P2Y_2R$-mediated LT-α expression contributes to salivary gland inflammation in IL-14αTG mice (Woods et al., 2018). Interestingly, LT-α levels are increased in the saliva, serum and salivary glands of SS patients, as compared to healthy individuals (Shen et al., 2010; Teos et al., 2015), and blockade of the LT-α receptor has been shown to reduce sialadenitis and improve the secretory function of the salivary gland in the IL-14αTG and NOD mouse models of SS (Gatumu et al., 2009; Shen et al., 2013). Lastly, unpublished observations from our lab indicate that expression of the *$P2Y_2R$* is increased in salivary gland-infiltrating B cells in NOD.H-2^{h4}, $CD28^{-/-}$, $IFN\gamma^{-/-}$ mice, as compared to B cells isolated from salivary glands of C57BL/6 control mice, and intraperitoneal administration of the selective $P2Y_2R$ antagonist AR-C118925 significantly attenuates sialadenitis and restores salivary gland function. In summary, these studies highlight the significant contributions of purinergic receptors to salivary gland inflammation and demonstrate their therapeutic potential for the treatment of human pro-inflammatory autoimmune diseases.

P2 RECEPTORS IN RADIATION-INDUCED HYPOSALIVATION

Radiation-induced salivary gland dysfunction is a common unintended side effect of radiotherapy in head and neck cancer patients, which causes xerostomia and hyposalivation that affects > 95% of these patients, > 73% of whom continue to suffer from months to years after completion of the radiotherapy (PDQ Supportive and Palliative Care Editorial Board, 2002; Dirix et al., 2006; Jensen et al., 2010; Pinna et al., 2015). Head and neck cancer patients routinely receive fractionated radiation treatment where the tumor region receives high radiation doses while salivary gland sparing techniques attempt to limit the radiation dose to 2 Gy/day (Eisbruch et al., 1999; Grundmann et al., 2009; Pfister et al., 2015). It is estimated that the tolerance dose for a 50% complications rate (TD50) for the parotid and submandibular glands is 28.4 and 39 Gy, respectively (Eisbruch et al., 1999; Li et al., 2007; Murdoch-Kinch et al., 2008). A number of factors including tumor grade, lymph node involvement and location of the tumor create scenarios where salivary gland sparing is not feasible and the tissue is exposed to higher radiation doses. Consequently, chronic hyposalivation and changes in the saliva electrolyte composition occur along with a reduction in pH that leads to alterations in oral microbial flora, increased incidence of dental carries and oral infections and difficulties with swallowing, digestion, and speech (Hu et al., 2013; Pinna et al., 2015).

Several groups have utilized rodent models to demonstrate that acute hyposalivation occurs immediately after IR, before the onset of overt gland damage, which is associated with sustained increases in the $[Ca^{2+}]_i$ (Coppes et al., 2005; Liu et al., 2013, 2017; Ambudkar, 2018). In contrast, chronic IR-induced salivary dysfunction results from ROS production, increased caspase-3 activity, disruption of store-operated Ca^{2+} entry (SOCE), cytoskeletal rearrangements, acinar cell apoptosis, sialadenitis and replacement of normal parenchyma with fibrotic tissue (Coppes et al., 2001; Radfar and Sirois, 2003; Teymoortash et al., 2005; Muhvic-Urek et al., 2006; Avila et al., 2009; Liu et al., 2013, 2017; Wong et al., 2018). One of the early responses to IR

is impairment of muscarinic receptor signaling (Coppes et al., 2000, 2005; Konings et al., 2005) required for saliva formation and aquaporin channel activity required for fluid secretion (Takagi et al., 2003). Furthermore, Avila et al. (2009), have demonstrated that radiation also causes a significant reduction in saliva-secreting acinar cells due to p53-dependent apoptosis. Thus, the overall mechanism of radiation-induced salivary gland hypofunction likely involves perturbations in muscarinic receptor signaling, apoptosis of saliva-producing acinar cells and irreversible tissue damage.

The P2X7R is highly expressed in salivary epithelium where its activation induces responses associated with IR-induced hyposalivation, including ROS production, caspase-3 activity, prostaglandin E_2 and ATP release, NLRP3 inflammasome activation with IL-1β release and salivary gland cell apoptosis (Woods et al., 2012; Khalafalla M.G. et al., 2017; Gilman et al., 2019). Thus, we recently explored the role of P2X7R activation in γ-radiation-induced hyposalivation. IR exposure induced ATP release from wild type mouse parotid gland epithelial cells (PGECs) that was attenuated by the P2X7R antagonist A-438079 and in PGECs isolated from $P2X7R^{-/-}$ compared to wild type mice (Gilman et al., 2019). Furthermore, systemic administration of A-438079 in γ-irradiated wild type mice conferred significant radioprotection to salivary glands and maintained saliva flow rates similar to non-irradiated mice at 3 and 30 days post-IR. This study also demonstrated that PGE_2 is secreted from wild type PGECs following γ-radiation that was reduced in $P2X7R^{-/-}$ PGECs or following A-438079 pretreatment of wild type PGECs (Gilman et al., 2019). Prostaglandins modulate inflammatory responses by altering cytokine production and secretion in macrophages (Ricciotti and Fitzgerald, 2011; Aoki and Narumiya, 2012). The signaling pathway downstream of cyclooxygenase-2 (COX-2), the rate-limiting enzyme that converts arachidonic acid into prostaglandins (Chandrasekharan and Simmons, 2004), has been shown to contribute to the IR-induced bystander effect in other cell types (Zhou et al., 2005; Chai et al., 2013; Kobayashi and Konishi, 2018) and P2X7R activation has been shown to induce arachidonic acid release from rat SMG ductal cells (Alzola et al., 1998). These findings suggest that P2X7R antagonists provide radioprotection by attenuating the damaging tissue response to IR-induced release of alarmins, including ATP and PGE_2.

P2 RECEPTORS IN SALIVARY GLAND REGENERATION

While most current treatments for salivary gland dysfunction target expansion of residual salivary acinar cells to repair damaged tissue, regenerative therapy with stem cells is a novel and promising therapeutic approach to replace damaged salivary glands (Carpenter and Cotroneo, 2010; Lombaert et al., 2017; Ogawa and Tsuji, 2017). Several studies have identified and characterized subsets of endogenous salivary progenitor cells that can be exploited to promote tissue regeneration (Lombaert et al., 2008; Chibly et al., 2014, 2018; Pringle et al., 2016; Emmerson et al., 2018; Weng et al., 2018). The use of modified fibrin hydrogels (Nam et al., 2019a), layered sheets of isolated salivary gland cells released from thermoresponsive culture dishes (Nam et al., 2019b) and salivary organoid cultures generated from embryonic pluripotent stem cells (Tanaka et al., 2018) have been explored as regenerative therapies for damaged salivary glands. Tissue engineering of 3-dimensional (3-D) primary HSG cultures for transplantation into afflicted patients represents another regenerative strategy to restore salivary gland function (Lombaert et al., 2017). Because primary human salivary gland cells undergo loss of cell-specific protein expression and biological function when cultured in a monolayer (Jang et al., 2015), development of 3-D culture strategies using Matrigel (Feng et al., 2009; Maria et al., 2011), collagen-Matrigel (Joraku et al., 2007; Pringle et al., 2016), hyaluronic acid-based hydrogels (Pradhan-Bhatt et al., 2013) and magnetic 3-D levitation (Ferreira et al., 2019) has been explored to maintain salivary gland cell function in culture. Indeed, transplantation of 3-D cultured, primary human salivary gland cells has been shown to ameliorate radiation-induced salivary gland dysfunction in mice (Pringle et al., 2016).

Rodent salivary glands have been shown to possess a high capacity to regenerate following the ligation or obstruction of the main excretory ducts of the gland, where ligated salivary glands initially become inflamed before glandular atrophy occurs through TGF-β-induced fibrosis and Fas ligand-induced epithelial cell apoptosis (Burford-Mason et al., 1993; Ahn et al., 2000; Takahashi et al., 2004, 2005, 2007; Carpenter et al., 2007; Woods et al., 2015). Following de-ligation, residual cells in damaged salivary glands can regenerate the gland through proliferation, migration and self-organization (Takahashi et al., 1998; Man et al., 2001; Kishi et al., 2006; Aure et al., 2015), thereby restoring salivary gland function, i.e., increasing the secretion rate of saliva with a normal ion and protein composition (Scott et al., 1999; Osailan et al., 2006). Concurrent with these glandular changes, functional $P2Y_2R$ expression, which is very low under homeostatic conditions, is robustly increased in salivary epithelial cells in response to ductal ligation and $P2Y_2R$ expression returns to basal low levels following de-ligation and subsequent recovery of the salivary gland (Ahn et al., 2000; El-Sayed et al., 2014). These findings are in agreement with previous studies demonstrating $P2Y_2R$ upregulation in epithelial cells in response to tissue damage and inflammation (Turner et al., 1997; Schrader et al., 2005; Degagne et al., 2009; Woods et al., 2018), suggesting that the $P2Y_2R$ is an important component in the repair and regeneration of damaged salivary glands.

Previous studies have demonstrated a role for the $P2Y_2R$ in corneal epithelial wound healing by increasing cell migration (Boucher et al., 2010), in liver regeneration by stimulating hepatocyte proliferation (Tackett et al., 2014), in cardiac regeneration by stimulating cardiac progenitor cell proliferation (Khalafalla F.G. et al., 2017) and in intestinal epithelial cell tubulogenesis (Ibuka et al., 2015). Activation of $P2Y_2Rs$ in the HSG cell line also induces the transactivation, homodimerization and autophosphorylation of the EGFR, a receptor tyrosine kinase known to be crucial for salivary gland branching morphogenesis and development (Miyazaki et al., 2004; Patel et al., 2006; Mizukoshi et al., 2016). This process in salivary epithelial and endothelial cells involves ADAM10/17-dependent proteolytic cleavage induced by $P2Y_2R$ activation that causes the release

TABLE 1 | Expression and function of purinergic receptors in salivary glands.

Purinergic receptor	Cell or tissue type	Salivary gland function	References
P2X7	Rat parotid acinar cells	Mediates eATP-induced Ca^{2+} entry	Soltoff et al., 1990; Dehaye, 1993; McMillian et al., 1993, 1987
		Mediates eATP-induced plasma membrane permeabilization and large pore formation	Gibbons et al., 2001
	Rat submandibular acinar cells	Induces plasma membrane permeabilization and large pore formation	Alzola et al., 2001
		Inhibits carbachol- and substance P-induced mobilization of intracellular Ca^{2+}	Hurley et al., 1993; Metioui et al., 1996
		Increases phospholipase A2-dependent secretion of arachidonic acid and kallikrein	Alzola et al., 1998
	Rat submandibular acinar and ductal cells	Mediates eATP-induced Ca^{2+} entry and increases membrane Cl^- conductance	Lee et al., 1997
	Mouse parotid acinar cells	Modulates carbachol-induced Ca^{2+} mobilization	Novak et al., 2010
		Mediates eATP-induced Ca^{2+} entry, Ca^{2+}-induced Ca^{2+} release, and exocytosis	Bhattacharya et al., 2015, 2012
		Mediates eATP-induced membrane anion conductance	Reyes et al., 2008
	Mouse parotid acinar and ductal cells	Mediates eATP-induced Ca^{2+} entry and membrane conductance; cell-specific channel assembly properties	Li et al., 2003
		Mediates γ-radiation induced eATP and PGE_2 release	Gilman et al., 2019
	Mouse submandibular acinar and ductal cells	Mediates eATP-induced apoptosis, ROS production, NLRP3 inflammasome assembly and IL-1β release	Woods et al., 2012; Khalafalla M.G. et al., 2017
	Ex vivo mouse submandibular gland	Mediates eATP-induced fluid secretion and inhibits carbachol-induced fluid secretion	Nakamoto et al., 2009
	In vivo mouse salivary glands	Modulates carbachol-induced saliva secretion	Pochet et al., 2007; Novak et al., 2010
P2X4	Rat parotid acinar cells	Mediates eATP-induced Ca^{2+} entry	McMillian et al., 1993
	Mouse parotid acinar cells	Mediates eATP-induced Ca^{2+} entry and exocytosis; potentiated by increased cAMP levels	Bhattacharya et al. 2012; 2015
		Mediates eATP-activated membrane currents; functional interaction with P2X7 receptor	Casas-Pruneda et al., 2009
	Mouse submandibular ductal cells	Mediates eATP-induced Ca^{2+} entry	Pochet et al., 2007
	Human parotid acinar cells	Mediates eATP-induced Ca^{2+} entry; potentiated by increased cAMP levels	Brown et al., 2004
$P2Y_1$	Rat submandibular acinar and ductal cells	Mediates nucleotide-induced $[Ca^{2+}]_i$ increase; decreased activity in aged animals	Park et al., 1997
		Mediates nucleotide-induced $[Ca^{2+}]_i$ increase and ERK1/2 phosphorylation; differential coupling to $G\alpha_{14}$ and $G\alpha_{q/11}$ during development	Baker et al., 2006
$P2Y_2$	Rat parotid cell line ParC10	Mediates eUTP-induced increase in short-circuit current and Cl^- efflux	Turner et al., 1998a
	Rat submandibular acinar and ductal cells	Mediates eUTP-induced increase in membrane Cl^- conductance	Lee et al., 1997; Zeng et al., 1997
		Increased expression and eUTP-induced $[Ca^{2+}]_i$ increase during short-term culture	Turner et al., 1997
	In vivo rat submandibular glands	Increases CFTR-mediated Cl^- reabsorption to modify saliva ion content	Ishibashi et al., 2008
	Mouse submandibular acinar and ductal cells	Mediates eUTP-induced cell aggregation and migration through EGFR transactivation	El-Sayed et al., 2014
	In vivo mouse submandibular glands	Increased expression and eUTP-induced $[Ca^{2+}]_i$ increase during salivary gland inflammation	Schrader et al., 2005; Ahn et al., 2000; Woods et al., 2018
	Human salivary gland (HSG) cell line	Mediates UTP-induced IP_3 production, $[Ca^{2+}]_i$ increase and K^+ efflux	Yu and Turner, 1991
		Potentiates cell regulatory volume decrease in response to hypotonic stress	Kim et al., 1996
		Increases vascular cell adhesion molecule expression	Baker et al., 2008
		Mediates eUTP-induced EGFR phosphorylation and induces EGFR and ErbB3 heterodimerization	Ratchford et al., 2010

eATP, extracellular ATP; eUTP, extracellular UTP; PGE_2, prostaglandin E_2; CFTR, cystic fibrosis transmembrane conductance regulator; EGFR, epidermal growth factor receptor.

of cell surface-bound EGFR ligands as well as the Src kinase-dependent transactivation of growth factor receptors through the binding of Src to SH3 binding motifs in the $P2Y_2R$ intracellular domain (Liu et al., 2004; Seye et al., 2004; Ratchford et al., 2010). In HSG cells, $P2Y_2R$ activation also induces the heterodimerization of EGFR and ErbB3, another member of the EGFR family (Ratchford et al., 2010). ErbB3 has an inactive kinase domain that requires heterodimerization with EGFR to respond to its ligand, neuregulin, which then stimulates the ERK/MAPK signaling pathway to promote cell proliferation, migration, and differentiation (Patel et al., 2006; Ratchford et al., 2010).

Integrins are transmembrane cell surface receptors that interact with extracellular matrix components, including laminin (Nishiuchi et al., 2006), fibronectin (Bharadwaj et al., 2017) and collagen (Tuckwell and Humphries, 1996), intracellular cytoskeletal proteins and other cell surface receptors (Legate et al., 2009) that are crucial components in the salivary gland regeneration process (Wei et al., 2007; El-Sayed et al., 2014). Hence, the bi-directional nature of integrin signaling regulates many physiological processes relevant to salivary gland regeneration, including cell proliferation, polarity, migration, and adhesion (Legate et al., 2009). Through its extracellular RGD domain, the $P2Y_2R$ can bind directly to integrins (e.g., $\alpha_v\beta_{3/5}$) and allow for nucleotide-induced $P2Y_2R$-mediated activation of integrin signaling pathways, including Rho and Rac GTPase activation that regulate cytoskeletal rearrangements (Erb et al., 2001; Wang et al., 2005). The extracellular ligand for the $\alpha_5\beta_1$ integrin is fibronectin, a well-known mediator of salivary gland morphogenesis (Sakai et al., 2003; Onodera et al., 2010), and we have previously demonstrated that UTP-induced $P2Y_2R$ activation also induces $\alpha_5\beta_1$ integrin-mediated migration, aggregation, and self-organization of dispersed salivary epithelial cells into acinar-like spheres (El-Sayed et al., 2014). These spheres resemble native acinar units of the salivary gland, possessing a lumen and organized expression of the tight junction protein ZO-1, and we have shown that the mechanism for $P2Y_2R$-mediated self-organization of salivary gland cells involves the activation of EGFR via the Cdc42 Rho GTPase pathway and subsequent downstream activation of ERK1/2 and JNK signaling pathways (El-Sayed et al., 2014). Thus, these studies suggest a promising role for unique structural motifs in $P2Y_2Rs$ that are highly relevant to cell-based regenerative therapy and bioengineering of salivary glands.

SUMMARY

Activation of purinergic receptors for extracellular nucleotides in the salivary glands modulates various physiological and pathophysiological functions (**Table 1**). The ATP-gated ionotropic P2X7 receptor in salivary acinar cells contributes to physiological salivary gland function by modulating muscarinic receptor-induced saliva secretion into the ductal lumen, whereas activation of ductal P2X7Rs modulates ion and protein content of saliva. P2X4R activation also contributes to saliva secretion through the formation of functional homotrimers and P2X4R/P2X7R heterotrimers in salivary gland epithelium, suggesting that P2XRs represent an integration point between canonical and non-canonical signaling pathways that regulate saliva flow and composition. $P2Y_1Rs$ also may contribute to salivary gland development through coupling to multiple G proteins resulting in diverse physiological responses. The ability of $P2Y_2R$ activation to stimulate increases in $[Ca^{2+}]_i$ and Cl^- flow across epithelial membranes suggests a role in saliva secretion, however, $P2Y_2R$ expression is negligible under normal steady-state conditions. The observed upregulation of $P2Y_2R$ expression during tissue stress and in response to P2X7R-induced IL-1β release suggest their significant role in salivary gland pathophysiology. Due to an increase in extracellular nucleotide release during tissue inflammation and dysregulation, nucleotide-induced activation of the interconnected P2X7R-$P2Y_2R$ signaling pathways likely modulates multiple immunological and tissue repair functions, including cell migration, growth factor receptor transactivation, integrin signaling, adhesion molecule upregulation, and cytokine release. Thus, P2X7R activation in salivary epithelium and upregulation of the $P2Y_2R$ with its unique structural domains likely regulate both salivary gland dysfunction and repair through the stimulation of these important pro-inflammatory processes.

In conclusion, purinergic receptors have emerged as promising therapeutic targets to promote physiological saliva flow, prevent salivary gland inflammation and enhance tissue regeneration required to reverse common causes of salivary gland dysfunction in humans, such as the autoimmune disease SS or the side effect of radiotherapy in head and neck cancer patients. Because purinergic receptors share common agonists and form heteromeric receptors with distinct pharmacologic profiles, unraveling the contribution of intracellular P2 receptor cross-talk to salivary gland dysfunction in animal models and humans will further define their therapeutic value in the treatment of salivary gland disorders. The continued development of high affinity P2R agonists and antagonists and the investigation of their safety and efficacy represent the next steps in the clinical translation of this promising P2 receptor research.

AUTHOR CONTRIBUTIONS

MK, LW, KJ, KF, and JC reviewed literature and drafted the manuscript. MK, LW, KJ, KF, JC, JJ, KL, HG, and GW critically revised, edited, and approved the manuscript.

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$P2Y_{12}$ Receptor Antagonist Clopidogrel Attenuates Lung Inflammation Triggered by Silica Particles

*Patricia Teixeira Santana†, Tatiana Luna-Gomes†, Marcos Vinicius Rangel-Ferreira, Augusto Shuiti Tamura, Carolyne Lalucha Alves Lima Da Graça, Mariana Nascimento Machado, Walter Araujo Zin, Christina Maeda Takiya, Debora Souza Faffe and Robson Coutinho-Silva**

Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

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***Correspondence:**
Robson Coutinho-Silva
rcsilva@biof.ufrj.br

† These authors have contributed equally to this work

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Silicosis is an occupational lung disease caused by inhalation of silica particles. It is characterized by intense lung inflammation, with progressive and irreversible fibrosis, leading to impaired lung function. Purinergic signaling modulates silica-induced lung inflammation and fibrosis through P2X7 receptor. In the present study, we investigate the role of $P2Y_{12}$, the G-protein-coupled subfamily prototype of P2 receptor class in silicosis. To that end, BALB/c mice received an intratracheal injection of PBS or silica particles (20 mg), without or with $P2Y_{12}$ receptor blockade by clopidogrel (20 mg/kg body weight by gavage every 48 h) – groups CTRL, SIL, and SIL + Clopi, respectively. After 14 days, lung mechanics were determined by the end-inflation occlusion method. Lung histology was analyzed, and lung parenchyma production of nitric oxide and cytokines (IL-1β, IL-6, TNF-α, and TGF-β) were determined. Silica injection reduced animal survival and increased all lung mechanical parameters in relation to CTRL, followed by diffuse lung parenchyma inflammation, increased neutrophil infiltration, collagen deposition and increased pro-inflammatory and pro-fibrogenic cytokine secretion, as well as increased nitrite production. Clopidogrel treatment prevented silica-induced changes in lung function, and significantly reduced lung inflammation, fibrosis, as well as cytokine and nitrite production. These data suggest that inhibition of $P2Y_{12}$ signaling improves silica-induced lung inflammation, preventing lung functional changes and mortality. Our results corroborate previous observations of silica-induced lung changes and expand the understanding of purinergic signaling in this process.

Keywords: silica particles, silicosis, purinergic receptors, $P2Y_{12}$ receptor, ADP, clopidogrel

INTRODUCTION

Silicosis is an occupational pneumoconiosis caused by inhalation of silica particles (free crystalline silicon dioxide), which remains a health problem for workers in many industries, including mining and civil construction (Kauppinen et al., 2000; Bhagia, 2012). New forms of exposure to silica particles are added to those classically implied as silica sources, such as dental supply factories using

quartz fillers (de la Hoz et al., 2004), dental technicians exposed to airborne residuals of silica (Ergün et al., 2014), jewelry workers exposed to silica-containing chalk molds used in casting (Murgia et al., 2007), denim sandblasters (Bakan et al., 2011; Akgun, 2016), and fabricators of artificial-stone worktops (Hoy et al., 2018). Silica particle deposition in lung parenchyma leads to intense inflammatory response, followed by progressive and irreversible lung fibrosis. Depending on the dose, silica may produce acute (accelerated silicosis) or various forms of chronic silicosis (Borges et al., 2002; Castranova et al., 2002; Hnizdo and Vallyathan, 2003; Langley et al., 2004; Rimal et al., 2005). Both high-dose acute and low-dose chronic silica exposures induce granulomatous changes in the lungs. The risk of disease is related to lifetime cumulative exposure and to the amount of inhaled crystalline silica, which, in turn, depends on the concentration and the size of respirable particles, as well as on individual susceptibility (Leung et al., 2012).

Purinergic signaling has been studied during silicosis and various inflammatory contexts, where it contributes to inflammatory exacerbation (Burnstock and Kennedy, 2011; Idzko et al., 2014; Savio et al., 2018). P2 class receptors are activated by extracellular nucleotides, such as ATP and ADP, and are subdivided into two subfamilies: P2X ligand-gated ion channels, and P2Y G-protein-coupled receptors (Burnstock and Kennedy, 2011). We previously demonstrated a significant role for P2X7 receptor as a regulator of silica-induced lung changes. Silica-induced ATP release activates P2X7 receptor, leading to the production of reactive oxygen species (ROS), inflammasome activation, and IL-1β release (Moncao-Ribeiro et al., 2014). By contrast, the role of P2Y receptors in silica inflammation is less well understood.

$P2Y_{12}$ receptor is mainly, but not exclusively, expressed on platelets. It mediates ADP-induced platelet aggregation, playing a central role in platelet biology (Kim and Kunapuli, 2011). More recently, $P2Y_{12}$ expression has been described also in immune cells, such as monocytes (Micklewright et al., 2018), dendritic cells (Ben Addi et al., 2010), and T lymphocytes (Wang et al., 2004). Furthermore, blocking $P2Y_{12}$ pathways alters T cell activation and changes the cell population (Vemulapalli et al., 2019). In the respiratory system, $P2Y_{12}$ receptor appears to contribute to inflammatory response, participating in allergic and non-allergic processes (Paruchuri et al., 2009; Shirasaki et al., 2013; Suh et al., 2016), as well as autoimmune disease processes (Domercq et al., 2018). Therefore, to better understand the role of purinergic signaling in silica-induced lung inflammation, we investigated the participation of $P2Y_{12}$ receptor in the onset of silicosis.

MATERIALS AND METHODS

Experimental Group

This study was approved by the Ethics Committee of the Health Sciences Center, Federal University of Rio de Janeiro (IBCCF164). All animals received humane care according to the Guiding Principles in the Care and Use of Laboratory Animals approved by the Council of the American Physiological Society. Male Balb/c mice (20–30 g, n = 36) were anesthetized with isoflurane (Isoforine®, Cristália, São Paulo, Brazil; 99% purity) and randomly divided into three groups, intratracheally injected with: phosphate-buffered saline (PBS, 100 μL) (CTRL group) or 20 mg of silica particles (approximately 80% 1–5 μm, Sigma, Chemical Co., St. Louis, MO, United States) without (SIL) or with (SIL + Clopi) clopidogrel (Plavix®, Sanofi-Aventis, Paris, France; 99% purity) treatment (20 mg/kg body weight by gavage each 48 h for 14 days). Animals were analyzed 14 days after PBS or silica administration.

Pulmonary Mechanics

Pulmonary mechanics were determined as previously described (Moncao-Ribeiro et al., 2014). Briefly, animals were sedated (diazepam 1 mg *i.p.* Valium®, Roche, Basel, Switzerland; 99% purity), anesthetized (pentobarbital sodium 20 mg/kg body weight *i.p.*, Nembutal®, Merck, Beijing, China; 99% purity), paralyzed (pancuronium bromide 0.1 mg/kg body weight *i.v.* Pancuron®, Cristália, São Paulo, Brazil; 99% purity), and mechanically ventilated (Samay VR15, Universidad de la República, Montevideo, Uruguay) with 100 breaths/min, tidal volume of 0.2 mL, flow of 1 mL/s, and positive end-expiratory pressure of 2.0 cmH_2O. The anterior chest wall was surgically removed, airflow (V′) was measured using a pneumotachograph (1.5-mm ID; length = 4.2 cm, distance between side ports = 2.1 cm) connected to the tracheal cannula, lung volume was obtained digital integration of the flow signal. The pressure gradient across the pneumotachograph and transpulmonary pressure were determined using Validyne MP-45-2 differential pressure transducers (Engineering Corp., Northridge, CA, United States). Lung airway resistance and stress relaxation/viscoelastic properties [resistive (ΔP1), viscoelastic/inhomogeneous (ΔP2), and total (ΔPtot) pressures, respectively], as well as lung elastance and ΔE, were determined by the end-inflation occlusion method, as previously described (Bates et al., 1985).

Pulmonary Histology and Histomorphometry

To verify silica-induced pulmonary lesions an additional group of mice subjected to the same experimental protocol described above was used. Fourteen days after PBS or silica injection, the left lungs were collected, fixed with 4% buffered formaldehyde solution, dehydrated, and embedded in paraffin. Sections (4-μm – thick) were cut and stained with hematoxylin-eosin for the description of qualitative alterations in the lung structure. Picrosirius red staining was performed to analyze collagen deposition. For histomorphometry quantifications, a computer-assisted image analysis system comprising a Nikon Eclipse E-800 microscope connected to a computer with a digital camera (Evolution, Media Cybernetics, Bethesda, MD, United States) coupled to Q-Capture 2.95.0 software (Silicon Graphic Inc., Milpitas, CA, United States) was used. High-quality photomicrographs (2048 × 1536 pixel buffer) were captured from non-overlapping lung areas. Data acquisition and analysis were

done without knowledge of the animal groups in all cases, by the same observer.

Twenty high-quality images of silicotic nodules at ×10 objective lens were analyzed per animal. The surface density of silicotic nodules was calculated as follows: (total nodular area × 100)/total image area. Neutrophil quantification in lung parenchyma was determined in 16 images/animal at ×40 objective lens (3–5 animals/group). Results were expressed as the total number of neutrophils/histological field. Collagen fiber deposition in lung parenchyma was quantified across 20 random non-coincident fields (×10 objective lens). Results were expressed as the percentage of surface density/total image area.

Nitric Oxide and Cytokine Measurements

The right lungs (from the same animals used for histological study) were macerated for nitric oxide (NO) and cytokine measurements in lung tissue homogenates. NO production was evaluated according to Griess method (Green et al., 1982), and fluorescence was measured at 570 nm wavelength (SpectraMax M, Molecular Devices, San Jose, CA, United States).

Cytokine concentrations (IL-1β, IL-6, TNF-α, and TGF-β) were determined by ELISA, with a detection limit of 50 pg/mL (R&D Systems, Minneapolis, MN, United States).

Statistical Analysis

One-way ANOVA, followed by Bonferroni post-test, was used to assess differences among groups. Student's *t*-test for independent samples, Chi-square test or Wilcoxon–Mann–Whitney test were applied whenever applicable. The level of significance was set at 5%.

RESULTS

Treatment With $P2Y_{12}$ Receptor Antagonist Reduced Weight Loss and Protected Animals From Death

Silica exposure induced weight loss along the 14 days after instillation, followed by a 50% reduction in survival rate. Clopidogrel treatment reduced weight loss (**Figure 1A**) and significantly improved animal survival (**Figure 1B**).

$P2Y_{12}$ Receptor Inhibition Led to Minor Changes in Lung Mechanics During Silicosis

As previously reported (Faffe et al., 2001; Moncao-Ribeiro et al., 2014), silica caused lung functional changes, increasing all lung mechanical parameters in relation to control. Inhibition of the $P2Y_{12}$ receptor by clopidogrel broadly improved lung function, including its resistive, elastic, and viscoelastic components (**Figure 2**).

$P2Y_{12}$ Receptor Inhibition Reduced Cellular Infiltration in Lung Parenchyma

Silica administration led to intense and diffuse lung parenchyma infiltration of inflammatory cells, such as neutrophils, granulomatous nodular formation, and collagen fibers deposition (**Figures 3A,B**), as previously described (Faffe et al., 2001; Moncao-Ribeiro et al., 2014). In contrast, clopidogrel-treated mice showed preserved areas of lung parenchyma with morphological delimitation of the alveolar septa, significantly fewer neutrophil infiltration, as well as collagen fibers deposition compared with the SIL group (**Figures 3C,D**).

P2Y12 Receptor Inhibition Reduced Silica-Induced Pro-Inflammatory and Pro-Fibrogenic Cytokine Secretion, as Well as Nitric Oxide Production

Silica instillation induced pro-inflammatory and pro-fibrogenic cytokine production in lung parenchyma – such as IL-6, IL-1β, TNF-α, and TGF-β – as well as increased nitrite production (**Figure 4**). Conversely, clopidogrel treatment significantly reduced silica-induced cytokine and nitrite secretion (**Figure 4**).

DISCUSSION

Purinergic signaling has been implicated in the development of several inflammatory diseases. We have previously demonstrated the role of P2X7 in silicosis (Moncao-Ribeiro et al., 2014; Luna-Gomes et al., 2015), an irreversible and progressive lung fibrotic disease characterized by long-lasting inflammation. The present study expands the understanding of purinergic signaling in silica-induced lung inflammation, evaluating the role of $P2Y_{12}$ receptor in a well-established murine model of silicosis. $P2Y_{12}$ receptor blockage prevented silica-induced lung inflammatory changes, improving lung function and animal survival. These results demonstrate that $P2Y_{12}$ signaling also participates in silicosis onset.

Nucleotides, such as ATP and ADP, are secreted during inflammation and bind to purinergic receptors, stimulating immune system cells in a paracrine and autocrine way (Idzko et al., 2014). Purinergic signaling is mediated by P2X (ATP-gated cation channels) and P2Y (G-protein-coupled) receptors (Burnstock, 2007; Shirasaki et al., 2013). P2X7 receptors have been shown important for leukocyte biology (Gu et al., 2000), as well as for silica-induced inflammation through NLRP3 inflammasome activation and IL-1β production (Moncao-Ribeiro et al., 2014; Luna-Gomes et al., 2015). On the other hand, $P2Y_{12}$ receptors, the prototype of P2Y subfamily, are important for platelet biology (Buvinic et al., 2002; Guns et al., 2005). Although mainly expressed on platelets, $P2Y_{12}$ expression was recently described in other cells of the immune system as well (Wang et al., 2004; Ben Addi et al., 2010; Junger, 2011; Micklewright et al., 2018). Indeed, $P2Y_{12}$ receptor blockage influences T cell activation and cell proliferation. The effect of ADP is specific for CD4 and CD8 T cells, while $P2Y_{12}$ antagonism alters these effects, suggesting functional expression of $P2Y_{12}$ on T cells (Vemulapalli et al., 2019). We showed previously that silica-induced inflammation increased macrophage, neutrophil, dendritic cell, as well as lymphocyte infiltration ($CD4^+$ and $CD8^+$) in lung parenchyma (Moncao-Ribeiro et al., 2014). The

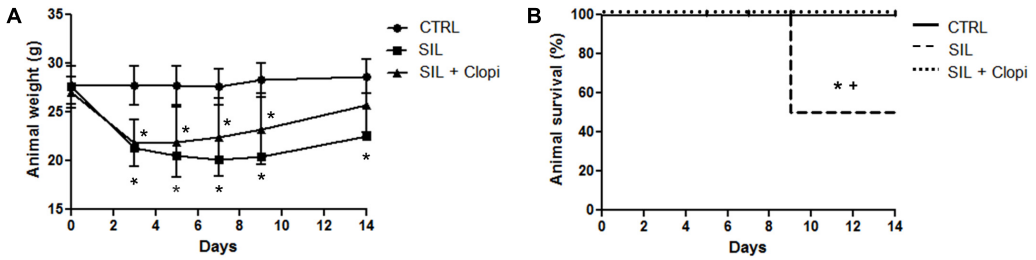

FIGURE 1 | $P2Y_{12}$ inhibition reduces silica-induced weight loss and prevents animal death. Body weight **(A)** and survival **(B)** were monitored along 14 days after PBS or silica (20 mg) injection without or with clopidogrel treatment (20 mg/kg) (CTRL, SIL, and SIL + Clopi groups, respectively). Values are mean ± SD of $n = 5–6$ animals/group. $^{*}p < 0.05$ compared with CTRL; $^{+}p < 0.05$ in relation to SIL + Clopi.

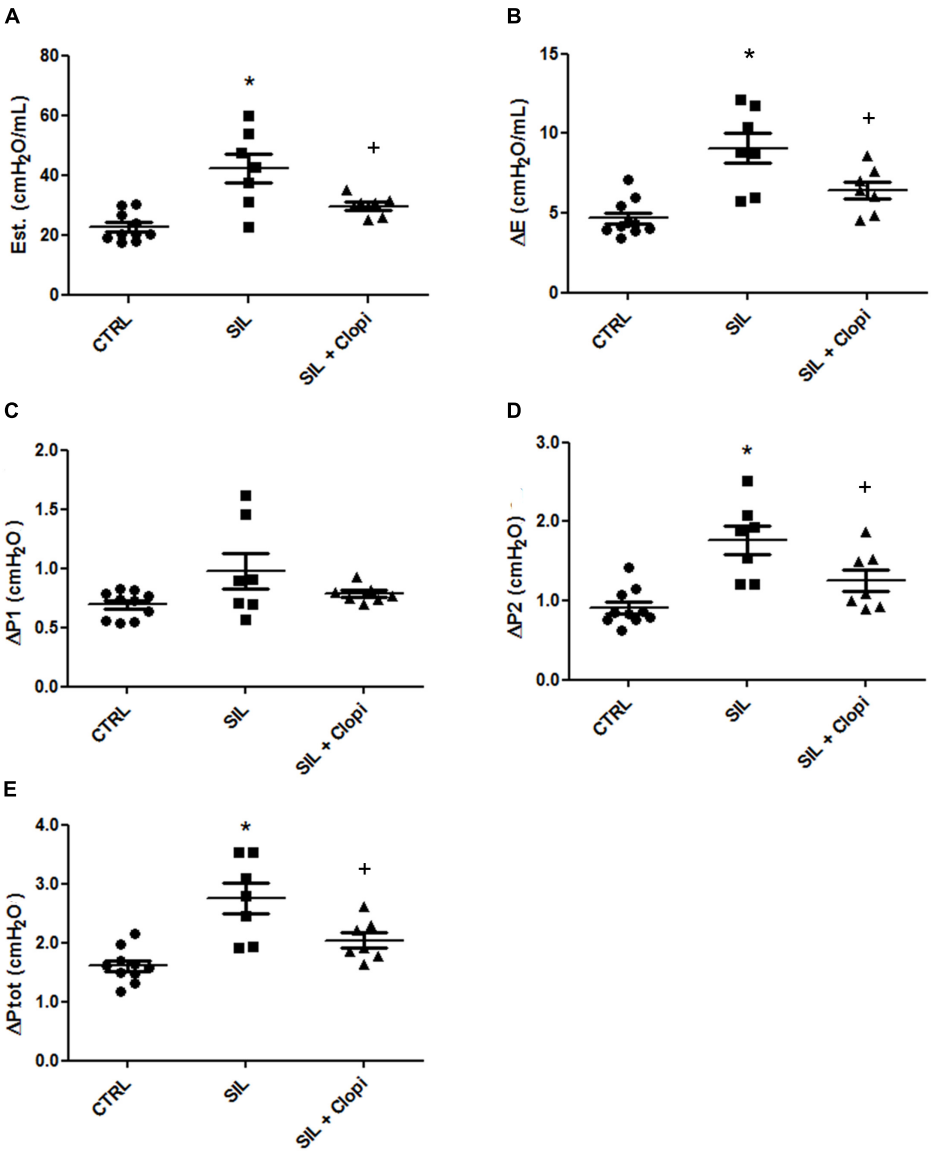

FIGURE 2 | $P2Y_{12}$ inhibition reduces silica-induced changes in lung mechanics. Lung static elastance (Est, **A**); viscoelastic component of elastance (ΔE, **B**); resistive (ΔP1, **C**), viscoelastic/inhomogeneous (ΔP2, **D**), and total (ΔPtot, **E**) pressures determined 14 days after PBS or silica (20 mg) injection without or with clopidogrel treatment (20 mg/kg) (CTRL, SIL, and SIL + Clopi groups, respectively). Values represent mean + SEM of 5–6 animals/group (10 determinations per animal). $^{*}p < 0.05$ in relation to control (CTRL); $^{+}p < 0.05$ in relation to silica (SIL).

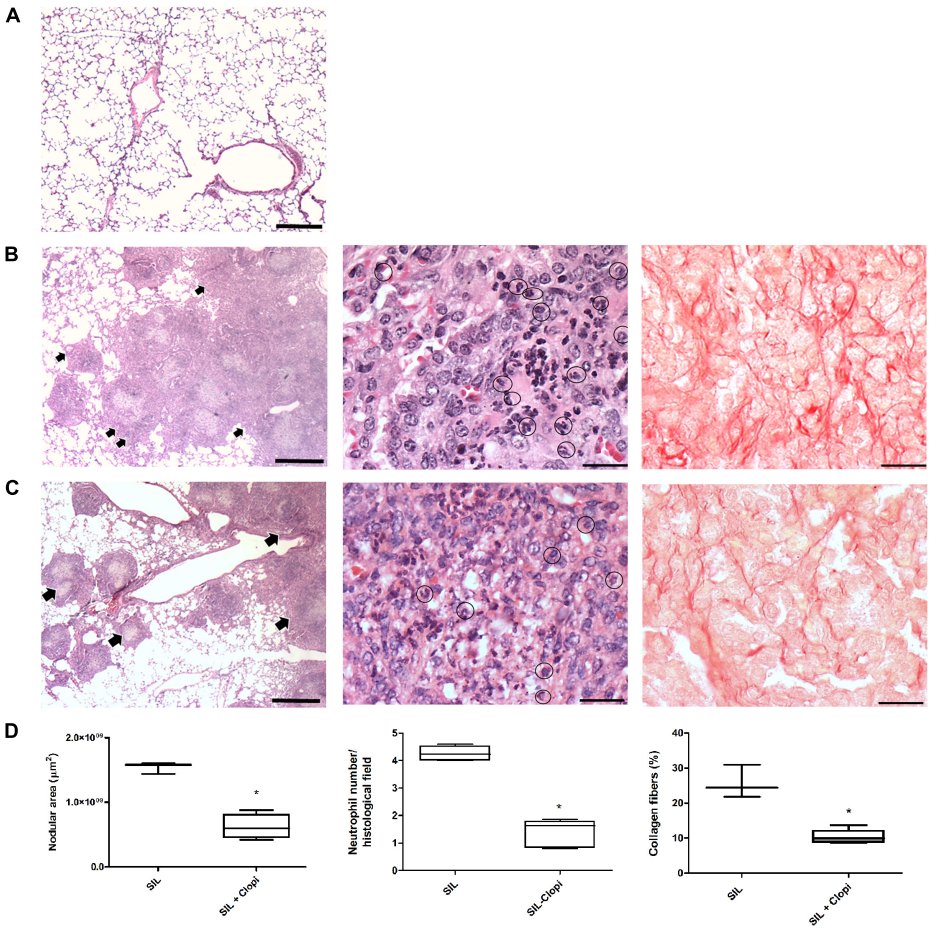

FIGURE 3 | $P2Y_{12}$ inhibition reduces silicotic lung inflammation and fibrosis. Representative lung parenchyma photomicrographs 14 days after PBS **(A)** or silica (20 mg) injection without **(B)** or with **(C)** clopidogrel treatment (20 mg/kg) (CTRL, SIL, and SIL + Clopi groups, respectively). Hematoxylin-eosin staining (left and central columns) and picrosirius staining (right column). **(D)** Quantification of nodular area, neutrophil infiltration, and collagen fibers (left, central, and right columns, respectively). Box plots show median values of 3–5 animals/group (16–20 images/animal) with respective minimum to maximum values. $^*p < 0.05$ in relation to silica (SIL). Arrows show inflammatory infiltrates. Bars: 1000 μm (left column), 50 μm (central and right columns).

present results now underline important participation of $P2Y_{12}$ signaling in inflammatory cell recruitment on the onset of the silicotic process. Our data support also recent evidence of the importance of $P2Y_{12}$ in biological functions of other immune cells rather than platelets. Recent data demonstrated a regulatory role for $P2Y_{12}$ receptor in regulating neutrophil influx into the lung during sepsis (Liverani et al., 2016). P2Y12 receptor antagonism also reduced inflammation in other inflammation models, including pancreatitis, ischemia-reperfusion, and LPS-induced lung injury (Hackert et al., 2009; Harada et al., 2011; Liu et al., 2011). It is worth note, however, that the $P2Y_{12}$ inhibitor clopidogrel – successfully used as antiplatelet medication to prevent thrombus formation in those at high risk – may also have $P2Y_{12}$ independent effects during inflammation, and neutrophils are the most likely target (Liverani et al., 2016). Therefore, we cannot exclude an additional direct effect of clopidogrel on reduced inflammation observed after $P2Y_{12}$ inhibition.

The immune physiopathology of silicosis involves the activation of inflammatory cells, especially alveolar macrophages. It has been shown that these cells contribute to increased lung oxidant secretion, as well as other inflammatory mediators, including interleukin 1β and tumor necrosis factor-alpha (TNF-α) (Jagirdar et al., 1996). NO is associated with inflammation and damage in asthma and LPS-induced inflammation (Belvisi et al., 1995; Moncao-Ribeiro et al., 2011; Liverani et al., 2014). NO also plays a crucial role in murine silicosis. Silica particle exposure activates macrophages to release NO (Moncao-Ribeiro et al., 2011). *In vivo* studies showed that mice exposed to silica develop exacerbated lung inflammation, while iNOS-deficient mice are more resistant to silica-induced inflammation (Srivastava et al., 2002). Previous studies showed that silica particle exposure induces TNF-α, IL-1β, and IL-6 secretion in lung parenchyma in a time-dependent manner (Vanhee et al., 1995; Moncao-Ribeiro et al., 2014). IL-1β is associated with cell recruitment, leading to neutrophil and eosinophil infiltration into lung tissue (Sims and Smith, 2010). In the context of silicosis, the lysis of alveolar macrophage releases cellular components into the extracellular environment, including IL-1β, promoting the recruitment of

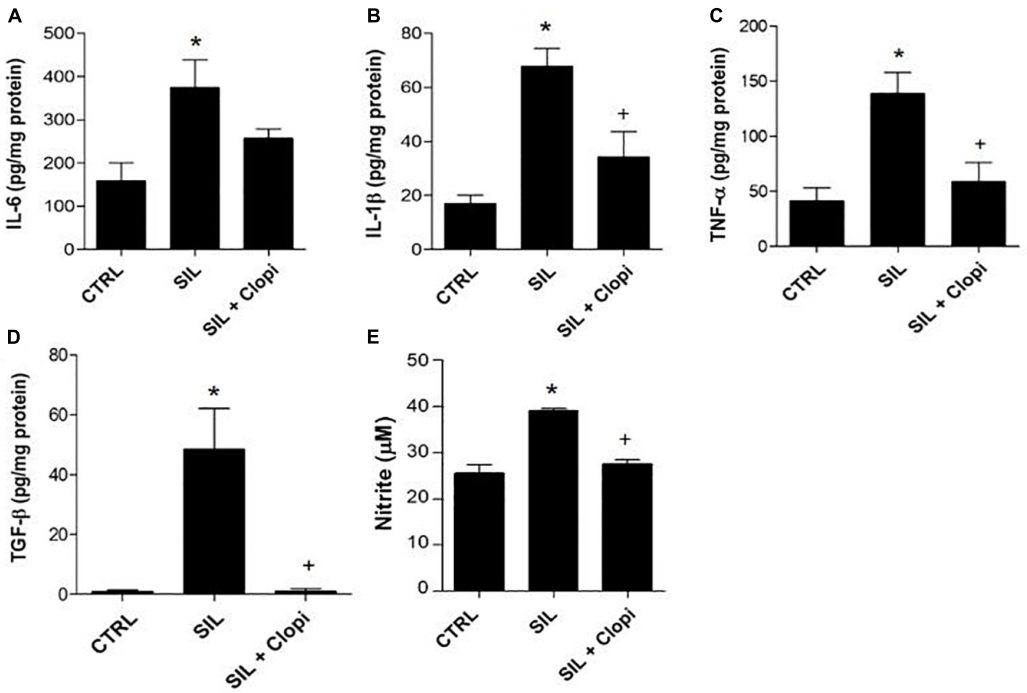

FIGURE 4 | $P2Y_{12}$ inhibition reduces silica-induced pro-inflammatory and pro-fibrotic cytokines, as well as nitrite production in lung parenchyma macerates. **(A)** IL-6, **(B)** IL-1β, and **(C)** TNF-α pro-inflammatory, and **(D)** TGF-β pro-fibrotic cytokines by ELISA. **(E)** nitrite production. Values are mean + SEM of 5–6 animals/group determined 14 days after PBS or silica (20 mg) injection without or with clopidogrel treatment (20 mg/kg) (CTRL, SIL and SIL + Clopi groups, respectively). $^{*}p < 0.05$ in relation to control (CTRL); $^{+}p < 0.05$ in relation to silica (SIL).

inflammatory cells into alveoli and endothelial walls (Moncao-Ribeiro et al., 2014). Purinergic signaling participates in IL-1β secretion by macrophages, as well as in NO production through P2X7 activation, as previously demonstrated by our group. On the other hand, $P2Y_{12}$ receptor does not participate in cytokine secretion (Vemulapalli et al., 2019), but its blockage significantly reduced NO and the pro-inflammatory mediators IL-6 and TNF-α, due to reduced cellular recruitment.

During the silicotic process, injured lung tissue is repopulated with fibroblasts, yielding excessive extracellular matrix deposition and fibrosis, followed by impairment of lung function (Willis and Borok, 2007). In murine models of lung fibrosis, IL-1β has been associated with collagen deposition, while IL-1β receptor blockage reduces pulmonary fibrosis caused by silica or bleomycin (Rimal et al., 2005). IL-6 also promotes pulmonary fibrosis after silica exposure, with excessive extracellular matrix proliferation (Le et al., 2014; Tripathi et al., 2010). In addition, TGF-β is a main pro-fibrotic mediator in remodeling after tissue injury (Fernandez and Eickelberg, 2012), as well as in the fibrotic process trigged by silica exposure (Jagirdar et al., 1996; Moncao-Ribeiro et al., 2014). TGF-β induces extracellular matrix remodeling, collagen production and fibroblast proliferation in the lung parenchyma. Once secreted, TGF-β has chemotactic and proliferative effects on fibroblasts (Sime and O'Reilly, 2001). It also stimulates the secretion of various proinflammatory and fibrogenic cytokines, including TNF-α, IL-13, and IL-1β, thereby increasing and perpetuating the fibrotic response in lung tissue (Fernandez and Eickelberg, 2012). Our results demonstrate that $P2Y_{12}$ inhibition significantly reduces TGF-β production, thus supporting a role for $P2Y_{12}$ signaling in silica-induced fibrosis through TGF-β modulation. Silica exposure impairs lung function – affecting its elastic, resistive and viscoelastic components. Lung functional changes are secondary to granuloma formation, alveolar collapse, as well as cellular infiltration in the lung parenchyma (Faffe et al., 2001; Moncao-Ribeiro et al., 2014; Cruz et al., 2016). Silica particle inhalation also promotes a fibrogenic response characterized by lung remodeling and replacement of damaged epithelial cells with collagen fiber deposition in the lung parenchyma (Honma et al., 2004; Willis and Borok, 2007). $P2Y_{12}$ receptor blockage reduced inflammation and lung remodeling significantly enough to prevent functional changes. Our data corroborate previous observations and expand the understanding of purinergic signaling in silica-induced lung changes. Finally, it is worth note that the animal model of silica-exposure used in the present study does not reproduce chronic silicosis. It does present, however, well-established functional and histological pulmonary changes 14 days after silica administration (Faffe et al., 2001; Borges et al., 2002). Silica-induced chronic lung fibrosis usually results from long-lasting inflammation. Therefore, a better understanding of $P2Y_{12}$ receptor role in acute inflammation would improve our knowledge about purinergic signaling in silicosis, opening new avenues to modify disease progression.

CONCLUSION

In conclusion, our results demonstrate that $P2Y_{12}$ receptor is involved in silicosis, probably via its immunomodulatory effects. These findings corroborate and expand previous observations of purinergic signaling participation in silica-induced lung changes. Identification of novel mechanisms involved in disease progression may help in the development of efficient therapies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Health Sciences Center, Federal University of Rio de Janeiro (IBCCF164).

AUTHOR CONTRIBUTIONS

PS, TL-G, and RC-S drafted the manuscript. MR-F, TL-G, and PS conducted and analyzed the data from all experiments. AT and CD conducted the cytokine measurement experiments. DF, WZ, CT, and RC-S contributed to the conception and design of the study, and revised the draft. All authors contributed to the manuscript revision, and approved the submitted version.

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REVIEW
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To Inhibit or Enhance? Is There a Benefit to Positive Allosteric Modulation of P2X Receptors?

Leanne Stokes, Stefan Bidula, Lučka Bibič and Elizabeth Allum*

School of Pharmacy, University of East Anglia, Norwich, United Kingdom

The family of ligand-gated ion channels known as P2X receptors were discovered several decades ago. Since the cloning of the seven P2X receptors (P2X1-P2X7), a huge research effort has elucidated their roles in regulating a range of physiological and pathophysiological processes. Transgenic animals have been influential in understanding which P2X receptors could be new therapeutic targets for disease. Furthermore, understanding how inherited mutations can increase susceptibility to disorders and diseases has advanced this knowledge base. There has been an emphasis on the discovery and development of pharmacological tools to help dissect the individual roles of P2X receptors and the pharmaceutical industry has been involved in pushing forward clinical development of several lead compounds. During the discovery phase, a number of positive allosteric modulators have been described for P2X receptors and these have been useful in assigning physiological roles to receptors. This review will consider the major physiological roles of P2X1-P2X7 and discuss whether enhancement of P2X receptor activity would offer any therapeutic benefit. We will review what is known about identified compounds acting as positive allosteric modulators and the recent identification of drug binding pockets for such modulators.

Keywords: P2X receptor, allosteric modulator, pharmacology, drug discovery, P2X4, P2X7

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University of Ferrara, Italy

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Günther Schmalzing,
RWTH Aachen University, Germany
Ralf Schmid,
University of Leicester,
United Kingdom

****Correspondence:***
Leanne Stokes
l.stokes@uea.ac.uk

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INTRODUCTION

Over the last decade we have seen new developments in pharmacological agents targeting P2X4, P2X7, and P2X3 receptors with some candidates entering clinical trials (Keystone et al., 2012; Stock et al., 2012; Eser et al., 2015; Matsumura et al., 2016; Timmers et al., 2018; Muccino and Green, 2019). Drug discovery for other P2X receptors such as P2X1 and P2X2 is somewhat slower with very few selective and potent drugs being identified (Burnstock, 2018). Advances in structural biology have helped move drug design for P2X receptors forward. Accompanying this is the advance in knowledge of the types of physiological responses controlled by this family of ion channels and clinical areas where such drugs may be therapeutically useful. Much emphasis has been placed on the development of antagonist agents and relatively little attention has been on the discovery or development of positive modulators. In this review, we take stock of all the evidence regarding the known physiological roles of the major P2X receptors and present what we currently know about pharmacological agents that can enhance ATP-mediated responses.

In receptor function and pharmacology, the word "allosteric" is commonplace. The historical use of this word is discussed by Colquhoun and Lape (2012) and the use of the term allosteric in current pharmacological terminology is used in the context of allosteric modulators, allosteric interactions, and allosteric transitions (Neubig et al., 2003). Allosteric transition describes the mechanism underlying receptor activation; following ligand binding to an orthosteric site, there is conformational communication through the protein to activate the biological response, for example, ion channel pore opening (Changeux and Christopoulos, 2016). Pharmacologically, multiple sites exist on receptor proteins where ligands can bind. While agonists bind at orthosteric sites, modulators act at distinct allosteric (other) sites and typically affect agonist action (Neubig et al., 2003). Allosteric drug interactions can have different outcomes, either positive, negative, or neutral/silent modulatory effects (Neubig et al., 2003; Changeux and Christopoulos, 2016). For positive allosteric modulators (PAMs), these can alter sensitivity to the agonist by shifting the concentration-response curve, alter agonist efficacy by increasing the maximum response, or alter gating kinetics of the ion channel by affecting activation or deactivation (Chang et al., 2010). At the molecular level, it is thought that PAMs reduce the energy barrier for gating thus making it easier for an ion channel to transition into an active, open state (Chang et al., 2010). These two different effects on the agonist actions have been classified by some as Type I (increasing the maximum response or efficacy) and Type II (shifting the concentration-response curve and thus altering agonist EC_{50} values) (Hackos and Hanson, 2017). These two effects may not always be separated with some PAMs exhibiting both effects (mixed Type I/II) (**Figure 1**). The therapeutic beauty of PAMs is their reliance on the presence of the endogenous agonist for activity. Therefore, in disease states where agonist-induced receptor signaling is perhaps defective, PAMs could help to bring those responses back into the "normal" range.

Other ligand-gated ion channels such as the Cys-loop family ($GABA_A$ receptor, glycine receptor, nicotinic acetylcholine receptor, and 5-HT_3 receptor) have many drug binding pockets which are well characterized for both PAMs and negative allosteric modulators (NAMs). The barbiturates and benzodiazepines act as PAMs at the $GABA_A$ receptor and are clinically used as sedatives, anti-convulsants, and anaesthetic agents (Solomon et al., 2019). Newer developments have seen multiple PAMs for α_7 nAchR advance into clinical trials for Alzheimer's disease, schizophrenia, and ADHD (Yang T. et al., 2017). PAMs for NMDA ionotropic glutamate receptors could be useful for disorders where their hypofunction is implicated (e.g., schizophrenia) (Yao and Zhou, 2017) and PAMs for AMPA ionotropic glutamate receptors could be useful in several cognitive disorders (Lee et al., 2016). For the P2X receptors, only ivermectin, a PAM with activity on P2X4, has been assessed in a pilot Phase 1 clinical trial for alcohol-use disorders (Roche et al., 2016).

We have recently discovered and characterized novel positive allosteric modulators that act on P2X7 and P2X4 receptors (Helliwell et al., 2015; Dhuna et al., 2019) and this has prompted us to ask questions about whether PAMs hold any therapeutic benefit for P2X receptors. We present here a review of the latest literature on P2X receptors focusing on the major identified subtypes P2X1, P2X2, P2X2/3, P2X4, and P2X7 with well-known physiological roles (P2X5 and P2X6 have no assigned physiological roles as homomeric receptors).

P2X1 RECEPTOR

The P2X1 receptor is a fast desensitizing ion channel activated by ATP, αβ-Me-ATP, βγ-Me-ATP, 2-MeSATP, BzATP, and Ap4A (North and Surprenant, 2000). Human P2X1 was cloned from urinary bladder RNA, and later from human platelets (Longhurst et al., 1996; Sun et al., 1998). Mackenzie et al. first recorded P2X1 currents from human platelets (Mackenzie et al., 1996) and P2X1-dependent Ca^{2+}signaling in platelet activation was characterized (Sage et al., 1997). In 1999, P2X1 was stated to have no significant role in platelet aggregation (Takano et al., 1999) however, a later study by Oury et al. demonstrated that P2X1 did act as a positive regulator of platelet responses (Oury et al., 2001). Further to this, a transgenic mouse over-expressing P2X1 in megakaryocytes demonstrated increased αβ-Me-ATP-sensitive Ca^{2+} responses *ex vivo* and more profound platelet

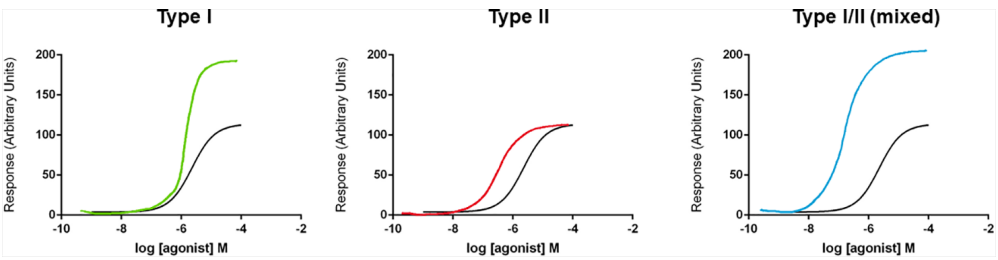

FIGURE 1 | Schematic diagram of types of positive allosteric modulator (PAM) effects on concentration-response curves. Three different types of PAM effect are displayed on concentration-response curves (for illustration purposes only, curves are hand-drawn). A Type I effect is defined as increasing the maximum response without a change in EC_{50} value. A Type II effect is defined as a left-ward shift in the concentration-response curve (and a reduction in the EC50 value) without affecting efficacy (maximum response). A mixed Type I/II effect is defined as both a left-ward shift in concentration-response curve and an increase in efficacy (maximum response).

shape changes to this agonist (Oury et al., 2003). Tests on these P2X1 over-expressing platelets revealed an increase in collagen-induced aggregation and in the transgenic mouse, an increase in fatal pulmonary thromboembolism was observed compared to wild-type mice (Oury et al., 2003). This work demonstrates that the expression level of P2X1 can modulate platelet aggregation responses. Other studies have investigated the synergy between P2X1 and P2Y1 GPCRs on platelets and it appears that P2X1 activation alone does not induce platelet aggregation (Jones et al., 2014) but that a synergistic activation of P2X1/P2Y1 enables full platelet aggregation. Ca^{2+} influx through P2X1 was deemed critical for this effect (Jones et al., 2014). It is therefore postulated that P2X1 acts as a coincidence detector for released nucleotides and can modulate responses through other platelet receptors (Grenegård et al., 2008; Jones et al., 2014) such as adrenaline and thrombin receptors (Jones et al., 2014) and FcγRIIα (Ilkan et al., 2018). This may be a crucial physiological role for P2X1 to amplify intracellular Ca^{2+}-dependent signaling *via* release of nucleotides in an autocrine loop (Ilkan et al., 2018). It is also suggested that P2X1 expressed on neutrophils can be involved in thrombosis (Darbousset et al., 2014) as $P2X1^{-/-}$ mice demonstrated increased polymorphonuclear (PMN) cell accumulation in a laser-injury model which reduced thrombus formation. Thrombosis was restored upon infusion of both platelets and PMNs from wild-type mice whereas infusion of platelets alone did not restore thrombus formation (Darbousset et al., 2014). This was confirmed by using NF449, a selective P2X1 antagonist, demonstrating abolishment of PMN recruitment to the site of injury. With the wealth of evidence showing that P2X1 contributes to platelet aggregation responses, any chronically applied pharmacological agent enhancing P2X1 Ca^{2+} influx in platelets could therefore cause an increased risk of thrombosis, particularly if a positive modulator affects the rate of channel desensitization. Alternatively, acute positive pharmacological modulation may enhance aggregation and clot formation and this may be useful in cases where patients were actively bleeding.

P2X1 is also known to play a role in smooth muscle contraction. ATP is released alongside noradrenaline from sympathetic nerves as a non-adrenergic non-cholinergic (NANC) neurotransmitter. This ATP acts on P2X1 receptors localised on postsynaptic smooth muscle cells (e.g., vas deferens) to contribute to the excitatory junction potential and contractile response (Kennedy, 2015). This work was originally pioneered by Geoffrey Burnstock leading to the accepted notion of purinergic neurotransmission (Burnstock, 2006). ATP is also released from parasympathetic nerves together with acetylcholine and acts on postsynaptic P2X1 in the urinary bladder to induce contractile responses (Kennedy, 2015). It is now thought that P2X1 is the predominant receptor in arterial, bladder, gut, and reproductive smooth muscle (Vial and Evans, 2001). In vascular smooth muscle P2X1 has a role in sympathetic nerve mediated vasoconstriction (Vial and Evans, 2002) and in the renal vasculature, P2X1 is implicated in the regulation of cortical and medullary blood flow by inducing vasoconstriction. In isolated kidneys this autoregulation increases vascular resistance and preglomerular microvascular regulation is thought to stabilize the glomerular filtration rate (Guan et al., 2007). $P2X1^{-/-}$ mice have an impairment in this protective autoregulatory behavior (Inscho et al., 2003). In hypertensive disorders, this renal autoregulation can be defective and purinergic receptors may contribute to the pathophysiology. For example, in Angiotensin-II mediated models of hypertension, there is an increase in vascular resistance causing a reduction in glomerular filtration rate. There is conflicting evidence regarding the expression of P2X1 following chronic administration of Angiotensin-II with some studies reporting an increase (Franco et al., 2011) and other studies reporting a reduction in P2X1 expression (Gordienko et al., 2015).

In the bladder, the purinergic component is likely to be P2X1 and a heteromeric form of P2X1 together with P2X4 (P2X1/4 heteromer) (Kennedy, 2015) and neurogenic contractions can be cholinergic and non-cholinergic. In bladder dysfunction, such as interstitial cystitis, there is an increase in the non-cholinergic mechanisms. In reproductive smooth muscle such as vas deferens, P2X1 induces contractile responses and $P2X1^{-/-}$ mice display defective contraction and are reported to have a deficit in male fertility (Mulryan et al., 2000). Male mice were shown to copulate normally and the reduction in fertility was due to a reduced number of sperm in the ejaculate rather than from sperm dysfunction (Mulryan et al., 2000). Contraction of the vas deferens to sympathetic nerve stimulation in $P2X1^{-/-}$ mice was reduced by 60% (Mulryan et al., 2000). Further studies have shown that the ectonucleotidase NTPDase 1 plays an important role in regulating the activity of P2X1 in the vas deferens by preventing chronic desensitization (Kauffenstein et al., 2014). It has been suggested that pharmacological modulation of P2X1 could be useful in the treatment of male fertility. Blockade of P2X1 may represent a novel target for male contraception but conversely, potentiating P2X1 activity may enhance male fertility associated with defective contraction. Such treatments would need to be tissue-specific to prevent side effects on other physiological responses described.

In terms of therapeutic interventions, enhancement of P2X1 responses could be beneficial in acute platelet aggregation and clot formation, or in boosting vas deferens contractile responses to enhance male fertility (although this would need to be tissue-restricted).

POSITIVE ALLOSTERIC MODULATORS OF P2X1

There are very few studies describing compounds that can potentiate P2X1 responses. Two compounds described as PAMs include MRS2219 and gintonin. MRS2219 is an analogue of PPADS shown to selectively enhance rat P2X1 expressed in *Xenopus oocytes* using two-electrode voltage clamp recordings (Jacobson et al., 1998). MRS2219 had an EC_{50} of 5.9 μM and had no effect on rat P2X2, rat P2X3, or rat P2X4 currents (Jacobson et al., 1998). Gintonin is a water-insoluble non-saponin component of ginseng, made of

carbohydrate (mostly glucose), lipids (linoleic acid, palmitic acid, oleic acid, lysophospholipids, phosphatidic acid), and amino acids (Choi et al., 2015) and mainly acts on lysophosphatidic acid (LPA) receptors (Im and Nah, 2013). Using a *Xenopus oocyte* expression system, gintonin was shown to potentiate human P2X1 responses (Sun-Hye et al., 2013) with a similar proposed mechanism to phosphoinositides such as PIP_2 (Bernier et al., 2008b). Since the exact component of gintonin responsible for P2X1 potentiation is unclear, this needs more investigation in order to be useful in drug discovery. Overall, more information is required on chemicals that can act as selective PAMs at P2X1.

P2X2 RECEPTOR

The P2X2 receptor is a non-desensitizing ion channel activated by agonists such as ATP, BzATP, and 2-MeSATP (North and Surprenant, 2000). P2X2 was first cloned from rat PC12 pheochromocytoma cells (Brake et al., 1994) and human P2X2 was subsequently cloned from pituitary tissue RNA (Lynch et al., 1999). There is evidence that P2X2 can exist as a homomeric receptor and as a heteromeric receptor in combination with P2X3 (Lewis et al., 1995).

P2X2 is expressed in the inner ear and is thought to play a role in the regulation of hearing. P2X2 mRNA expression increases from embryonic day 12 to postpartum day 8–12 when expression peaks, then decreases to adult levels (Housley et al., 1998). ATP levels in the endolymph are low, P2X2 is activated following exposure to loud noises and contributes to otoprotection whereby ATP is released and acts to reduce the endocochlear potential (Thorne et al., 2004). Part of the adaptive response to loud noise involves an upregulation of P2X2 expression in the rat cochlea (Wang et al., 2003). This upregulation in response to stressors was less in older mice suggesting that this may increase the susceptibility of older animals to noise-induced hearing loss (Telang et al., 2010). In 2013, a human study identified a mutation in *P2RX2* in a Chinese family with inherited progressive hearing loss (Yan et al., 2013). Characterization of this mutation in a HEK-293 cell heterologous expression system revealed that the Val 60 > Leu mutation in P2X2 was not able to respond to ATP (Yan et al., 2013). Individuals carrying the mutation developed severe hearing loss by the age of 20 (Yan et al., 2013). This study also used the $P2X2^{-/-}$ mouse to demonstrate that age-related hearing loss was much greater in these animals than those expressing P2X2 (Yan et al., 2013). In 2014, a second mutation in *P2RX2* was identified in an Italian family with hereditary hearing loss (Faletra et al., 2014). This mutation changed Gly 353 > Arg, a residue in the TM2 domain (Faletra et al., 2014). In 2015, a Japanese study identified a third mutation in *P2RX2* (Asn201 > Tyr) associated with severe hearing loss (Moteki et al., 2015). P2X2 is also thought to play a role in modulating vestibular function. $P2X2^{-/-}$ mice had impaired reflexes in response to sinusoidal rotation when compared to wild-type mice (Takimoto et al., 2018) and were more likely to slip when crossing a narrow beam (Takimoto et al., 2018). It may be advantageous to positively modulate P2X2 responses in the inner ear to increase otoprotection in response to any damage caused by loud noises. This may be of benefit in elderly individuals where P2X2 expression levels are reduced. Similarly, positive modulation of P2X2 may help with balance disorders (e.g., vertigo and Meniere's disease) and this angle may warrant further investigation.

P2X2 is known to be expressed throughout the hypothalamus and pituitary gland and is involved in the release of hormones such as arginine vasopressin but not oxytocin (Custer et al., 2012). The hypothalamus connects to secretory cells of the anterior pituitary which release chemicals such as luteinising hormone, thyroid stimulating hormone, adrenocorticotrophic hormone (ACTH), growth hormone, prolactin, and follicle-stimulating hormone. This system is intricately balanced and depends on feedback regulation. P2X2 has been directly linked to the enhancement of LH release from the pituitary (Zemkova et al., 2006). The hypothalamus also controls feeding and drinking behavior, reproductive behavior, and temperature regulation and P2X2 is expressed on neurons involved in the regulation of food intake (Collden et al., 2010). Other than regulating hormone secretion directly, P2X2 can play a neuromodulatory role by regulating the release of other neurotransmitters such as glutamate and GABA (Vavra et al., 2011) and in paraventricular neurons, P2X2 can modulate sympathetic activity (Ferreira-Neto et al., 2017). From a therapeutic viewpoint, disorders of the hypothalamus and pituitary gland typically include over-activation (e.g., Cushing's disease, tumors of NET) rather than under-activation and strategies are needed to limit excessive hormonal secretion. The impact of modulating P2X2 inputs in the hypothalamus is currently unknown.

In 2013, Cao et al. suggested that stimulating P2X2 receptors may be a potential therapeutic strategy for depressive disorders (Cao et al., 2013). Following chronic social defeat stress, mice were found to have lower ATP levels in the brain than control unstressed mice (Cao et al., 2013). Administration of ATP into ventricles (*i.c.v* injection) elicited an anti-depressant-like effect in the immobility test (forced swim test) and the non-hydrolysable ATP analogue, ATPγS, had a larger effect. Administration of ATP together with Cu^{2+} which is known to enhance P2X2 responses, reduced immobility and was interpreted as having an anti-depressant effect (Cao et al., 2013). The proposed mechanism involved ATP release from astrocytes acting on P2X2 receptors in the medial prefrontal cortex and a reduction in P2X2 expression using AAV-shRNA abolished the anti-depressant effect of ATP (Cao et al., 2013). This may provide an interesting mechanistic approach to developing novel anti-depressants, potentially by enhancing ATP release or by enhancing P2X2 signaling.

P2X2 AND P2X2/3 HETEROMERS

ATP was identified as a neurotransmitter involved in rodent taste perception and areas of the tongue areas involved in taste sensation (circumvallate and fungiform papillae) express both

P2X2 and P2X3 receptors (Bo et al., 1999). This was confirmed in a study using transgenic mouse models (Finger et al., 2005). Single gene knockout mice for P2X2 and P2X3 displayed reduced gustatory afferent nerve firing responses to some tastants, however, only in the P2X2/P2X3 double knockout mice were taste responses dramatically affected, losing responses to both sweet and bitter tastants (Finger et al., 2005). This suggested that the P2X2/3 heteromer was responsible for the gustatory neuron signaling to the gustatory cortex and that ATP was crucial for taste signaling. Release of ATP from type II cells, which do not synapse with the gustatory afferent neuron, also appears to be important in taste signaling. In P2X2/3 double knockout mice, tastants failed to release ATP (Huang et al., 2011). As P2X3 is only found in afferent neurons, this indicates a role for homomeric P2X2 receptors in the release of ATP from taste cells. Modulation of either homomeric P2X2 or heteromeric P2X2/3 receptors could therefore affect taste sensation and some P2X3 antagonists have been noted for their suppressive effect on taste in clinical trials (Muccino and Green, 2019).

In terms of therapeutic interventions, enhancement of P2X2 responses could be beneficial in otoprotection or in the modulation of mood. Further mechanistic research into the related physiology and pathophysiology is required to decide if such a pharmacological approach would be viable.

POSITIVE ALLOSTERIC MODULATORS OF P2X2

There is surprisingly little information about the pharmacology of P2X2, in particular human P2X2. In 2011, four derivatives of the anthraquinone dye Reactive Blue 2 were described as having positive allosteric modulator effects at rat P2X2 (Baqi et al., 2011). Reactive Blue 2 is a non-selective antagonist with effect at rat P2X2 and the derivative compound 51 (PSB-10129) increased the maximum response induced by ATP (Baqi et al., 2011). This could be classed as a PAM with Type I effect (**Table 1**). This work demonstrated that lipophilic substitution at certain positions could turn a negative modulator into a positive modulator. Two neurosteroids, dehydroepiandrosterone (DHEA) and progesterone, are known to positively modulate P2X2. DHEA can potentiate both homomeric P2X2 and heteromeric P2X2/3 in recombinant expression models (De Roo et al., 2003; De Roo et al., 2010). Conversely, progesterone potentiates ATP-induced currents in rat dorsal root ganglion neurons and P2X2-expressing HEK-293 cells (De Roo et al., 2010) but not in P2X2/3 expressing *Xenopus oocytes*, suggesting that it is selective for homomeric P2X2 (De Roo et al., 2010). Both of these neurosteroids increased the response to submaximal but not saturating concentrations of ATP suggesting that they affect potency of the agonist but not efficacy. Complete concentration-response experiments would be needed to confirm this, but these could be classed as PAMs with Type II effects (**Table 1**). Testosterone is an endogenous steroid with no potentiating activity at rat P2X2 (Sivcev et al., 2019). However, several synthetic 17β-ester derivatives of testosterone including testosterone butyrate and testosterone valerate, act as PAMs at P2X2 (Sivcev et al., 2019). The testosterone derivatives increased the sensitivity of P2X2 to ATP, reducing the EC_{50} (Sivcev et al., 2019), therefore these PAMs likely have Type II effects (**Table 1**). From this evidence, it is possible that steroids/neurosteroids can act as endogenous positive modulators of P2X2. This could be therapeutically useful, for example, some steroids have been used in treatment of sensorineural hearing loss and Meniere's disease, as reviewed in (Keiji et al., 2011).

TABLE 1 | Chemicals identified as having positive allosteric modulator activity at P2X receptors.

Drug name	Target Receptor	Predicted PAM effect (Type I, II, mixed)	Reference
MRS2219	P2X1 (rat)	Unknown	(Jacobson et al., 1998)
Gintonin	P2X1 (rat)	Unknown	(Sun-Hye et al., 2013)
PIP_2	P2X1 (rat)	Type I	(Bernier et al., 2008a)
PSB-10129	P2X2 (rat)	Type I	(Baqi et al., 2011)
DHEA	P2X2 (rat) P2X2/3 (rat)	Type II	(De Roo et al., 2003)
Progesterone	P2X2 (rat)	Type II	(De Roo et al., 2010)
Testosterone butyrate	P2X2, P2X4	Type II Mixed Type I/II	(Sivcev et al., 2019)
Ivermectin	P2X4 (rat) P2X4 (human) P2X7 (human)	Mixed Type I/II Mixed Type I/II	(Khakh et al., 1999) (Priel and Silberberg, 2004) (Nörenberg et al., 2012)
Abamectin	P2X4 (rat)	Unknown	(Asatryan et al., 2014)
Selamectin	P2X4 (rat)	Unknown	(Asatryan et al., 2014)
Moxidectin	P2X4	Unknown	(Huynh et al., 2017)
Cibacron blue	P2X4 (rat)	Unknown	(Miller et al., 1998)
Alfaxolone	P2X4 (rat)	Unknown	(Codocedo et al., 2009)
Allopregnanolone	P2X4 (rat)	Unknown	(Codocedo et al., 2009)
THDOC	P2X4 (rat)	Unknown	(Codocedo et al., 2009)
Ginsenoside CK	P2X7 (human), P2X7 (mouse) P2X4 (human)	Mixed Type I/II Mixed Type I/II Mixed Type I/II	(Helliwell et al., 2015) (Bidula et al., 2019a) (Dhuna et al., 2019)
Ginsenoside Rd	P2X7, P2X4	Unknown Unknown	(Helliwell et al., 2015) (Dhuna et al., 2019)
Clemastine	P2X7 (human)	Type II	(Norenberg et al., 2011)
Tenidap	P2X7 (mouse)	Mixed Type I/II	(Sanz et al., 1998)
Polymyxin B	P2X7	Mixed Type I/II	(Ferrari et al., 2004)
Garcinolic acid	P2X7 (human)	Unknown	(Fischer et al., 2014)
Agelastine	P2X7 (human)	Unknown	(Fischer et al., 2014)
GW791343	P2X7 (rat)	Mixed Type I/II	(Michel et al., 2008a)

A Type I positive allosteric modulator (PAM) effect increases efficacy whereas a Type II PAM effect increases sensitivity to agonist seen as a left-ward shift in the concentration response curve. A mixed Type I/II PAM effect represents both features.

P2X3 RECEPTOR

The P2X3 receptor is a rapidly desensitizing ion channel activated by agonists such as ATP, αβ-MeATP and 2-MeSATP (North and Surprenant, 2000). P2X3 receptors are expressed in

sensory neurons where they play a role in nociceptive transmission, taste sensation, bladder distension, and chemoreceptor reflexes (Fabbretti, 2019). P2X3 was cloned from dorsal root ganglion sensory neurons (Chen et al., 1995) and is expressed on afferent C-fibre nerve terminals in peripheral tissues as well as being expressed in central terminals of dorsal root ganglia, as reviewed in (Bernier et al., 2018). Consequently, P2X3 contributes to acute pain signaling and potentially to chronic pain pathways as well (Bernier et al., 2018). P2X3 is expressed in carotid body neurons that regulate the chemoreflex sympatho-excitatory response controlling blood pressure (Pijacka et al., 2016). During the pathophysiology associated with hypertension, P2X3 upregulation can contribute to hyperreflexia and high blood pressure (Pijacka et al., 2016). A recent role for P2X3 has been postulated in chronic cough and airway sensitization due to expression on airway vagal afferent neurons (Abdulqawi et al., 2015; Ford et al., 2015). Continuing this theme of regulating sensory activity, P2X3 is expressed on gustatory sensory neurons and is responsible for taste signaling to the gustatory cortex (Vandenbeuch et al., 2015). As already mentioned, taste signaling involves homomeric P2X3 as well as heteromeric P2X2/3 receptors (Finger et al., 2005). Finally, P2X3 is also documented to have a role in the sensory control of bladder volume. Afferent neurons innervating the bladder express P2X3 and P2X3$^{-/-}$ mice display a reduced bladder voiding frequency (Cockayne et al., 2000). Collectively, this information about the major physiological roles of P2X3 does not present a strong case whereby potentiating ATP-responses at this receptor would be therapeutically useful. There are no known PAMs acting on P2X3 receptors. However, to date nothing is known about the presence of loss-of-function mutations in P2X3 and whether this could be linked to hypo-function of bladder reflexes, for example.

P2X4 RECEPTOR

P2X4 is a moderately desensitizing ion channel which is activated by ATP, ATPγS, and BzATP (North and Surprenant, 2000). First cloned from rat brain (Soto et al., 1996), P2X4 is widely expressed in the central nervous system, cardiovascular, epithelial, and immune systems. One of the first identified physiological roles for P2X4 was in the cardiovascular system where shear stress-induced ATP release was demonstrated to activate P2X4 on endothelial cells to induce a vasodilatation response (Yamamoto et al., 2000; Yamamoto et al., 2006). Endothelial cells deficient in P2X4 display no flow-regulated Ca^{2+} response or nitric oxide production (Yamamoto et al., 2006). Blood pressure measurements were higher in P2X4$^{-/-}$ mice and the adaptive flow-dependent vascular remodeling response to carotid artery ligation was impaired similar to chronic flow-induced changes in the eNOS$^{-/-}$ mouse (Yamamoto et al., 2006). In humans, a role for P2X4 in regulating flow-dependent vascular tone is postulated and a rare loss-of-function polymorphism was associated with increased pulse pressure (Stokes et al., 2011). It is also thought that P2X4 in the heart could be cardioprotective since cardiac-specific over-expression of P2X4 in mice protected against heart failure (Yang et al., 2014; Yang et al., 2015). In vascular endothelial cells of the brain, P2X4 can also be activated by shear stress and can promote release of osteopontin, a neuroprotective molecule in ischaemic situations (Ozaki et al., 2016). P2X4 was required for ischaemic tolerance in a middle cerebral artery occlusion model of ischaemic stroke (Ozaki et al., 2016).

P2X4 is expressed in epithelial tissues such as salivary glands and bronchiolar epithelium. In the bronchioles, P2X4 is thought to maintain the beating of cilia in the mucus layer, helping to clear the airways of pathogens (Ma et al., 2006). A role has also been described in lung surfactant secretion from alveolar type II epithelial cells (Miklavc et al., 2013). In T lymphocytes, P2X4 can affect T cell activation and migration (Woehrle et al., 2010; Ledderose et al., 2018). In monocytes/macrophages, P2X4 has been linked to release of the chemokine CXCL5 (Layhadi et al., 2018) and the killing of *E. coli* bacteria (Csóka et al., 2018). In the latter study, macrophages taken from the P2X4$^{-/-}$ mouse failed to kill bacteria in response to ATP (Csóka et al., 2018). Potentiation of P2X4 with ivermectin enhanced killing of bacteria and in a mouse model of sepsis, ivermectin improved survival (Csóka et al., 2018).

In the central nervous system, P2X4 is widely expressed on neurons and its role here was recently reviewed (Stokes et al., 2017). Development of a transgenic mouse with a red fluorescent tdTomato under the control of the *P2RX4* promoter confirmed the widespread distribution of P2X4 in the central nervous system (Xu et al., 2016a). In neurons, P2X4 regulates synaptic transmission (Rubio and Soto, 2001; Sim et al., 2006; Baxter et al., 2011) including modulation of GABA release (Xu et al., 2016a). In terms of regulating behavior, P2X4$^{-/-}$ mice exhibit an increased intake of ethanol (Khoja et al., 2018) and this has led to much research on the role of P2X4 in alcohol-use disorders. Treatment with ivermectin counteracts the inhibitory effect of ethanol on P2X4 and can influence the intake of alcohol (Yardley et al., 2012; Franklin et al., 2014). P2X4$^{-/-}$ mice also demonstrate a defect in sensorimotor gating due to dysregulation of dopamine neurotransmission (Khoja et al., 2016). In this study, ivermectin was shown to enhance L-DOPA induced motor behavior suggesting that positive modulation of P2X4 may be a useful adjunct strategy for Parkinson's disease (Khoja et al., 2016).

Finally, one of the well-known roles for P2X4 involves pathological signaling contributing to neuropathic pain. P2X4 contributes to microglial activation and regulates the release of BDNF which can affect local neurotransmission in the dorsal horn of the spinal cord. Studies have shown that P2X4$^{-/-}$ mice are protected against neuropathic pain (Coull et al., 2005; Ulmann et al., 2008). This work has led to an intensive effort to find antagonists of P2X4 that could be used in the treatment of chronic pain states. Any development of PAMs for therapeutic use would need to be tested for adverse effects on pain states.

POSITIVE ALLOSTERIC MODULATORS OF P2X4

One of the pharmacologically defining features of P2X4 is potentiation by ivermectin (IVM) (Khakh et al., 1999), a

derivative of avermectin B1, a macrocyclic lactone produced by *Streptomyces avermitilis*. Through its action on glutamate-gated chloride channels in nematode worms (Cully et al., 1994), IVM is mostly known as a broad-spectrum anti-parasitic agent (Fisher and Mrozik, 1992). IVM also potentiates mammalian $GABA_A$ receptors (KrůŠek and Zemkova, 1994) and α_7-nicotinic acetylcholine receptors (Krause et al., 1998). At P2X4, IVM increases the amplitude of the ATP-induced current at P2X4 with an EC_{50} of ~ 0.25 μM (Priel and Silberberg, 2004; Gao et al., 2015), shifts the EC_{50} for ATP and it changes the desensitization of the P2X4 response (Khakh et al., 1999; Priel and Silberberg, 2004). Therefore, IVM has mixed TypeI/II effects (**Table 1**). IVM may also potentiate the heteromeric P2X4/P2X6 receptor but does not affect P2X2, P2X3, (rodent) P2X7 receptors, or P2X2/3 heteromers (Khakh et al., 1999). Although the crystal structures of closed and ATP-bound state of P2X4 have been solved (Kawate et al., 2009; Hattori and Gouaux, 2012), the IVM-bound structure of P2X4 remains unknown. Priel and Silberberg noted that extracellular application was required for IVM modulation of P2X4 suggesting that IVM does not interact with the intracellular domains (Priel and Silberberg, 2004). It is suggested that IVM most likely partitions into membrane where the lactone ring interacts with the TM domains of P2X4 at the protein-lipid interface. There is also a suggestion that IVM could also affect the trafficking and recycling of P2X4 (Stokes, 2013). Scanning alanine mutagenesis of TM1 and TM2 confirmed that residues near the extracellular surface of the plasma membrane are critical for IVM action (Jelinkova et al., 2006; Silberberg et al., 2007; Asatryan et al., 2010; Popova et al., 2013). Critically, these residues lie either in the extracellular domain (Trp50, Thr57, Ser69, Val60, and Val61) or in the TM2 domain (Asn338, Ser341, Gly342, Leu346, Gly347, Ala349, and Ile356). Asatryan et al. showed that certain amino acids at the interface of the ectodomain and TM2 (Trp46, Trp50, Asp331, Met336) are also involved in determining the selectivity of IVM for P2X4 (Asatryan et al., 2010). Furthermore, the residues lining the edge of the lateral portals are also important (Rokic et al., 2010; Samways et al., 2012; Rokic et al., 2014; Gao et al., 2015). Molecular docking studies have provided important insights and confirmed some of the experimental findings (Latapiat et al., 2017; Pasqualetto et al., 2018).

In various models of disease IVM-dependent increased P2X4 activity might affect alcohol intake, sensorimotor gating, and dopamine-induced motor behavior (Bortolato et al., 2013; Khoja et al., 2016; Khoja et al., 2018; Khoja et al., 2019) implicating P2X4 as a novel drug target for the treatment of alcoholism and psychiatric disorders. IVM has also been shown to have an anti-cancer effect; it kills breast cancer cells through potentiating P2X4/P2X7 signaling (Draganov et al., 2015).

Apart from IVM, other members of the avermectin family that affect P2X4 function are abamectin (ABM), selamectin (SEL), and moxidectin (MOX). ABM is structurally similar to IVM, and similarly potentiated the ATP-induced P2X4 currents. However, at concentrations higher than 3 μM, ABM induced P2X4 responses in the absence of ATP (Asatryan et al., 2014). This may indicate that ABM can act as a direct agonist at higher concentrations. Moreover, in the same concentration range as IVM, ABM was able to antagonize the inhibitory effect of ethanol (100 mM) (Asatryan et al., 2014). In contrast to ABM, SEL is structurally diverse to IVM and was less effective at potentiating P2X4. SEL displayed a lack of efficacy in attenuating the inhibitory effects of ethanol (Asatryan et al., 2014). Lastly, MOX does not possess any saccharide moieties which might add to the increased lipophilicity and a faster penetration across the blood-brain barrier. Similar to IVM and ABM, MOX potentiated the P2X4-mediated currents in *Xenopus* oocytes at 0.5–1 μM and decreased the inhibitory effects of 25 mM (but not 50 mM) ethanol on P2X4 (Huynh et al., 2017). Consequently, this supports the use of avermectins as potential drugs to prevent and treat alcohol use disorders.

Cibacron blue, an anthraquinone sulfonic acid derivative, can potentiate rat P2X4 receptors (Miller et al., 1998). Low concentrations (3–30 μM) resulted in a 4-fold increase in ATP responses however, when tested at 100 μM, cibacron blue was inhibitory at rat P2X4 (Miller et al., 1998). This molecule might represent a novel pharmacophore for the structure-based design of novel allosteric ligands. Similar to P2X2, P2X4 can be modulated by neurosteroids. Alfaxolone, allopregnanolone, and 3α, 21-dihydroxy-5α-pregnan-20-one (THDOC) potentiate rat P2X4 responses in *Xenopus oocytes* and at high concentrations both alfaxolone and THDOC could gate the receptor (Codocedo et al., 2009). The mechanism of potentiation was not investigated in detail but the active neurosteroids could increase response to 1 μM ATP suggesting they may increase receptor sensitivity to agonist (Codocedo et al., 2009). A study found that testosterone 17β-ester derivatives such as testosterone butyrate and testosterone valerate could enhance P2X4 responses (Sivcev et al., 2019) by increasing receptor sensitivity to agonist (mixed Type I/II effect).

Recently, our lab identified ginsenosides of the protopanaxdiol series as positive allosteric modulators at P2X7 and P2X4 receptors (Helliwell et al., 2015; Bidula et al., 2019b; Dhuna et al., 2019). By using a plethora of techniques, including fluorescent YOPRO-1 dye uptake assays in stable cell lines over-expressing human P2X4, calcium assays, and electrophysiology, we demonstrated that two ginsenosides, CK and Rd, show ~2-fold potentiation of ATP-responses at P2X4 (Dhuna et al., 2019) which could be classed as a mixed Type I/II effect (**Table 1**). Enhancement of P2X4 is less than enhancement of P2X7 and our docking studies have predicted that while the interacting amino acid residues are similar in both receptors, subtle differences in the binding pocket might modify the way these ginsenosides bind to P2X4 (Dhuna et al., 2019). However, this may also provide novel pharmacophore information for development of selective PAMs.

In terms of therapeutic interventions, enhancement of P2X4 responses could be beneficial in hypertension (to reduce blood pressure *via* vasodilation), sepsis, Parkinson's disease, or in alcohol-use disorders. Thus, more research is justified to investigate PAMs and to determine their mechanisms of action both *in vitro* and *in vivo*.

P2X7 RECEPTOR

P2X7 is a non-desensitizing ion channel activated by ATP and BzATP (North and Surprenant, 2000). First cloned in 1996 (Surprenant et al., 1996), P2X7 is expressed in immune cells such as monocytes, macrophages, NK cells, lymphocytes, and neutrophils (Di Virgilio et al., 2017) and has predominantly been characterized by the intracellular signaling pathways that it regulates (Bartlett et al., 2014). P2X7 requires high concentrations of ATP for activation and displays a somewhat unique secondary pore-forming phenomena allowing movement of organic molecules across the cell membrane. The physiological function (and substrates) of this secondary pore pathway is currently unclear, however, it is likely to play a role in many P2X7 signaling events (Di Virgilio et al., 2018). Activation by high concentrations of ATP is consistent with its role in inflammation, where ATP can be released from stressed or damaged cells and functions as a damage-associated molecular pattern (DAMP) (Di Virgilio et al., 2017). Often, activation of P2X7 at these inflammatory sites can be detrimental and this may contribute to the pathophysiology of a plethora of inflammatory disorders. Conversely, it is possible that P2X7 activation may be beneficial in the defence against intracellular pathogens and cancerous cells.

P2X7 is a known regulator of immune cell mediator secretion. Multiple studies have demonstrated secretion of cytokines from the IL-1 family (IL-1β, IL-1α, IL-18) in response to P2X7-dependent activation of the NLRP3-caspase-1 inflammasome (Giuliani et al., 2017). Other cytokines such as those relying on cleavage by metalloproteinases (e.g., TNF-α) are also released following P2X7 activation as well as other cell surface proteins (e.g., L-selectin, VCAM-1, CD23, and CD14) which are shed (Pupovac and Sluyter, 2016). The particular cytokines released by P2X7 may depend on the cell type under examination, for example, in T lymphocytes, P2X7 can contribute to IL-2 production and secretion (Yip et al., 2009). Current knowledge may only be the tip of the iceberg as other immune cell types have not been rigorously examined. P2X7 can also contribute to the regulation of various caspase-dependent and -independent cell death pathways, including autophagy, necrosis, pyroptosis, and apoptosis, governing the homeostatic turnover of cells and modulating immunity to pathogens (Di Virgilio et al., 2017). Although the major physiological roles of P2X7 may be complex to pin down, it is clear that this receptor is involved in inflammation and infection. For a comprehensive overview of the pathophysiological roles of P2X7 in this context, please refer to (Di Virgilio et al., 2017; Burnstock and Knight, 2018; Savio et al., 2018).

P2X7 activation is important in the defence against intracellular bacteria such as *Chlamydiae*, *Porphyromonas gingivalis*, and *mycobacteria* species. P2X7 promotes the acidification of intracellular organelles, phospholipase D activation and decreases bacterial load (Coutinho-Silva et al., 2001; Coutinho-Silva et al., 2003; Darville et al., 2007). Consequently $P2X7^{-/-}$ mice are more susceptible to vaginal infection by *Chlamydiae* (Darville et al., 2007). P2X7 plays an important role in defence to *P. gingivalis*, the causative agent of periodontitis, *via* regulation of inflammasome activation (Choi et al., 2013; Hung et al., 2013; Park et al., 2014). The role of P2X7 in mycobacterial infections appears to be strain specific. On the one hand, loss-of-function in P2X7 may contribute to enhanced susceptibility to pulmonary and extra-pulmonary tuberculosis in humans (Fernando et al., 2007). P2X7 participates in the elimination of the intracellular bacteria *via* phospholipase D activation and host cell apoptosis (Fairbairn et al., 2001; Placido et al., 2006; Fernando et al., 2007; Singla et al., 2012; Areeshi et al., 2015; Wu et al., 2015). Conversely, mice infected with hypervirulent mycobacterial strains cannot effectively control the infection and P2X7 contributes to the severity of inflammation and propagation of bacterial growth (Amaral et al., 2014). With such hypervirulent strains, mice deficient in P2X7 were better protected against the infection (Amaral et al., 2014).

A role for P2X7 in the immune response to parasites *Leishmania amazonensis*, *Toxoplasma gondii*, *Plasmodium falciparum* (Salles et al., 2017), and *Entamoeba histolytica* (Mortimer et al., 2015) is also becoming clear. Macrophages infected by *L. amazonensis* can reduce their parasitic load *via* the P2X7-dependent production of the mediator leukotriene B4 (LTB_4) (Chaves et al., 2009; Chaves et al., 2014). Again, $P2X7^{-/-}$ mice were more susceptible to infection (Figliuolo et al., 2017). P2X7 activation can drive the elimination of *T. gondii via* the production of ROS, acidification of intracellular organelles (Correa et al., 2010; Moreira-Souza et al., 2017), and secretion of pro-inflammatory cytokines (Miller et al., 2011; Miller et al., 2015; Correa et al., 2017; Huang et al., 2017). Therefore, for parasitic infections, enhancing P2X7 responses may be therapeutically beneficial.

In models of infection, it is less clear how P2X7 affects outcomes in cases of sepsis. In a murine model of sepsis, $P2X7^{-/-}$ mice had a better chance of survival (Santana et al., 2015; Wang et al., 2015). Pharmacological inhibition using the P2X7 antagonists A-740003 or Brilliant Blue G, resulted in increased survival, downregulating inflammation and maintaining mucosal barrier integrity (Greve et al., 2017; Savio et al., 2017; Wu et al., 2017). A risk genotype of human P2X7 containing a known gain-of-function haplotype (P2X7-4.1 in (Stokes et al., 2010)) was increased in a cohort of sepsis patients (Geistlinger et al., 2012). Recent work shows P2X7 activation in human monocytes compromised subsequent NLRP3 inflammasome activation by bacteria and contributed to mitochondrial dysfunction (Martinez-Garcia et al., 2019). Impairment of NLRP3 was associated with increased mortality in sepsis patients (Martinez-Garcia et al., 2019) suggesting that P2X7 activation plays a detrimental role in sepsis. In the same study, the murine CLP model was used to test the role of activation of P2X7 *in vivo* prior to induction of sepsis and the authors documented an increased mortality (Martinez-Garcia et al., 2019) However, opposing studies using the murine model suggest that P2X7 could be protective within sepsis and demonstrated increased mortality in $P2X7^{-/-}$ mice (Csoka et al., 2015). This issue of the role P2X7 plays during sepsis needs further investigation for further progress to be made.

P2X7 has been implicated in the immune response to several viruses including; vesicular stomatitis virus (VSV), influenza virus, dengue virus, and HIV. In the case of VSV and dengue virus, P2X7 plays a beneficial role, with ATP-induced signaling resulting in decreased viral replication (Correa et al., 2016; Zhang et al., 2017). Conversely, evidence points towards a detrimental role for P2X7 in influenza and HIV infections. In the case of influenza, P2X7 deficiency protected against a lethal dose of the virus due to a reduction in inflammatory mediators and reduced neutrophil recruitment (Leyva-Grado et al., 2017). More recently, administration of the P2X7 antagonist AZ11645373 or probenecid [an approved drug known to inhibit P2X7 (Bhaskaracharya et al., 2014)], improved survival and recovery to pathogenic influenza infection in a murine model (Rosli et al., 2019). For HIV infection, pharmacological inhibition of P2X7 could limit replication of the virus within macrophages, and prevent virion release (Hazleton et al., 2012; Graziano et al., 2015). With viral infections, enhancing P2X7 responses may only be beneficial in certain cases and much more work is needed to fully understand potential therapeutic interventions.

The role of P2X7 in anti-fungal immunity is currently under-explored but studies have reported that P2X7 is not involved in scavenging *Candida albicans* and in the production of IL-1β in response to yeast infection (Hise et al., 2009; Perez-Flores et al., 2016). Xu et al., demonstrated that invariant natural killer T (iNKT) cells release ATP and induce Ca^{2+} signaling in dendritic cells, which stimulates the production of prostaglandin E2, recruitment of neutrophils, and reduced *C. albicans* infection (Xu et al., 2016b). A more recent study showed that P2X7 was critical for the induction of adaptive immune responses to *Paracoccidioides brasiliensis* and survival (Feriotti et al., 2017).

P2X7 is also expressed in glial cells within the central nervous system including microglia, oligodendrocytes, astrocytes, and there is some (often debated) evidence for expression in neurons. In the CNS, more P2X7 plays potential physiological roles in neuronal axonal growth and modulation of neurotransmitter release but also participates in neuroinflammation (Bartlett et al., 2014). Under pathological conditions or following damage to the CNS, a significant amount of ATP can be released which contributes to neuroinflammation. It is predominantly activation of P2X7 on microglia that stimulates the production of pro-inflammatory mediators and ROS. This neuroinflammation combined with an increase in cell death stimulates an environment whereby extracellular ATP concentrations are further enhanced, stimulating more cell death, including the death of neurons. Dysregulated P2X7 activation has therefore been touted as a key contributor to the pathophysiology of Alzheimer's disease, Parkinson's disease, and multiple sclerosis, among others. Genetic ablation of P2X7 dampens neuroinflammation and enhances the clearance of amyloid-β plaques (Mclarnon et al., 2006; Ryu and Mclarnon, 2008; Ni et al., 2013). Such neuroinflammatory responses may also be involved in psychiatric disorders, as reviewed in (Bhattacharya and Biber, 2016). Indeed, a gain-of-function haplotype of human P2X7 has been repeatedly linked to various psychiatric conditions including bipolar disorder, major depressive disorder, and anxiety disorders (Czamara et al., 2018; Deussing and Arzt, 2018). Current drug development programs are focused on testing CNS penetrant P2X7 antagonists for psychiatric conditions (Bhattacharya and Ceusters, 2020).

In the cardiovascular system, P2X7 participates in inflammation, cell metabolism, and cell death and therefore impacts ischemic heart disease, stroke, and vascular diseases such as atherosclerosis, hypertension, thrombosis, and diabetic retinopathy. P2X7 activation can contribute to cardiac dysfunction in myocardial infarction due to its role in inflammation which can facilitate sympathetic sprouting and arrhythmia (Lindholm et al., 1987; Yang W. et al., 2017). Notably, activation of P2X7 by the synthetic agonist BzATP can upregulate the secretion of nerve growth factor (NGF), which may be linked to enhanced sympathetic hyper-innervation and sprouting (Yin et al., 2017). P2X7 is upregulated at the site of infarct and can promote the activation of the NLRP3 inflammasome and the release of inflammatory IL-1β within the ventricles (Yin et al., 2017). Inhibiting P2X7 was demonstrated to promote cardiac survival, suppress T cell mediated immune responses, and limit the risk of rejection (Vergani et al., 2013). P2X7 generally contributes to excessive inflammation in the vasculature and is implicated in several vascular diseases *via* IL-1β production and production of matrix metalloproteases (MMPs) which contributes to the pathophysiology of atherosclerosis (Lombardi et al., 2017).

In the lung, P2X7 is a potential target for lung hypersensitivity associated with chronic inflammatory responses. Targeting P2X7 may control IL-1β-induced lung fibrosis and silicosis (Moncao-Ribeiro et al., 2014). Inhibiting P2X7 on dendritic cells and eosinophils could be beneficial in the treatment of allergic asthma, and the anti-histamine, oxatomide, has been suggested to be a P2X7 antagonist (Yoshida et al., 2015). Further, P2X7 has been associated with pulmonary oedema and with emphysema, the latter linked to the inhalation of cigarette smoke inducing ATP release (Lucattelli et al., 2011). P2X7 is connected to the recruitment of inflammatory cells to the lung during injury, particularly neutrophils, which further enhance lung injury. In this case, deletion or inhibition of P2X7 appeared to be protective within the lung. Therefore, with both cardiovascular and lung disorders, enhancement of P2X7 responses would not likely be of any advantage and most research is focused on testing P2X7 inhibitors.

In bone, P2X7 is involved in osteogenesis (Sun et al., 2013) and the development of mature osteoblasts (Gartland et al., 2001; Ke et al., 2003; Panupinthu et al., 2008). In these cells P2X7 participates in functions such as production of lipid mediators, induction of transcription factors, propagation of intercellular calcium signaling between osteoblasts and osteoclasts, and intracellular signaling in response to fluid shear stress (Gartland et al., 2001; Jorgensen et al., 2002; Liu et al., 2008; Okumura et al., 2008; Panupinthu et al., 2008; Gavala et al., 2010). This positive role of P2X7 in the maintenance of bone strength is supported by studies utilizing mesenchymal stem cells (MSCs) taken from post-menopausal women, where bone mineralization and osteogenic

differentiation were impaired (Noronha-Matos et al., 2014). Notably, administration of BzATP *in vitro* could restore these functions, indicating an important role for P2X7 in driving the formation of bone. P2X7 has been suggested to be involved in differentiation of osteoclasts (Barbosa et al., 2011) and the generation of multinucleated cells, however some evidence from $P2X7^{-/-}$ mice shows that this is a redundant process not solely reliant upon P2X7 (Gartland et al., 2003; Ke et al., 2003; Agrawal et al., 2010). Treatment of osteoclasts with BzATP or high ATP to stimulate P2X7 can increase bone resorption and this effect is lost in $P2X7^{-/-}$ mice (Jiang et al., 2000; Armstrong et al., 2009; Hazama et al., 2009). Miyazaki et al. demonstrated that bone resorption relies on intracellular (ATP) and mitochondrial function (Miyazaki et al., 2012). In this study treatment of bone marrow-derived osteoclasts with extracellular ATP resulted in decreased survival and resorption (Miyazaki et al., 2012). The differences between may be due to species specific differences or genetic variation, but a fine balance between P2X7 activation/inactivation must be maintained to ensure optimal osteoclast function (Donnelly-Roberts et al., 2009; Bartlett et al., 2014).

Skeletal muscle is required for numerous structural and biological functions within the body. When muscles are stimulated, they release small amounts of ATP which propagates intracellular Ca^{2+} signaling and downstream biological effects. However, when muscles are damaged, much larger concentrations of ATP are released, triggering an inflammatory response. An acute inflammatory response is essential for muscle repair and regeneration, but prolonged inflammation can result in muscular dystrophies (Tidball and Villalta, 2010). P2X7 expression is increased in the muscles of Duchenne's muscular dystrophy patients and in murine models of muscular dystrophy (Young et al., 2012). P2X7 can contribute to sterile inflammation by promoting the release of inflammatory mediators from dystrophic muscles (Rawat et al., 2010) or contribute to deregulated homeostasis in dystrophic muscles (Young et al., 2015). In the MDX model of muscular dystrophy, P2X7 deficiency reduced dystrophic symptoms such as decreased muscle structure and increased inflammation, whilst promoting expansion of T regulatory cells known to suppress dystrophic muscle damage (Sinadinos et al., 2015). Surprisingly, cognitive and bone improvements were also noted in these animals (Sinadinos et al., 2015). As for many of the disorders linked to excessive inflammation, enhancement of P2X7 responses in this context would be predicted to be detrimental.

Finally, the role of P2X7 in cancer development and progression will be considered. A feature of some tumor cells are their high levels of P2X7 expression, which can mediate cell proliferation, or cell death depending upon the type of tumor, the variant of P2X7 expressed and potentially, the cellular environment. Tumors often produce high concentrations of extracellular ATP within the tumor core which would enable P2X7 signaling (Burnstock and Knight, 2018). P2X7 antagonists have been suggested as potential anti-metastatic agents by reducing tumor cell proliferation. Conversely, it is thought that activating P2X7 on tumor cells could result in cell death.

First of all, considering the role of P2X7 in tumor cell proliferation, expression of P2X7 on tumor cells is associated with accelerated tumor growth (Adinolfi et al., 2012). γ-irradiation can induce the release of ATP from B16 melanoma cells, which results in proliferation and tumor growth (Hattori et al., 2012). Inhibition of P2X7 with AZ10606120 reduced proliferation of human pancreatic duct adenocarcinoma and human neuroblastoma cells *in vitro* (Amoroso et al., 2015; Giannuzzo et al., 2016). Furthermore, AZ10606120 reduced neuroblastoma tumor growth in nude mice (Gomez-Villafuertes et al., 2015). P2X7 can contribute to the metastasis of human lung cancer cells, and P2X7 inhibition significantly decreased the migration of cancer cells transplanted into immunodeficient mice (Takai et al., 2014; Schneider et al., 2015). Emodin, a natural product antagonist of P2X7, could reduce the invasiveness of a highly invasive breast cancer cell line and ATP could elicit an increase in cell migration and metastasis in another breast cancer cell line (Jelassi et al., 2013; Xia et al., 2015). P2X7 expression is being used post-operatively as a prognostic indicator for survival in renal cell carcinoma patients (Liu et al., 2015). Expression of a non-pore functional P2X7 (nfP2X7) was found in pathological specimens from prostate cancer patients and was not observed in normal patients suggesting this as a possible biomarker of prostate cancer (Slater et al., 2004). A more recent study suggests nfP2X7 is broadly expressed on many tumor cells (Gilbert et al., 2019). While it is unclear which splice variant encodes nfP2X7, antibodies recognizing this different form of P2X7 have been tested in a Phase I safety and tolerability trials for basal cell carcinoma (Gilbert et al., 2017). P2X7 plays a deleterious role in osteosarcoma and can contribute to cancer-induced bone pain (Giuliani et al., 2014; Falk et al., 2015). With gliomas, P2X7 activation is linked to an increase in inflammation, intracellular calcium signals, and tumor cell migration (Morrone et al., 2016).

P2X7 may play a role in the host immune response to tumor cells. In 2015, Adinolfi et al. reported that tumor progression was accelerated in mice lacking P2X7 (Adinolfi et al., 2015). Expression of P2X7 on host immune cells was critical for controlling the anti-tumor immune response (Adinolfi et al., 2015). In $P2X7^{-/-}$ mice, an immunocompromized tumor infiltrate was characterized with few $CD8^+$ T cells and an increased number of T regulatory cells (De Marchi et al., 2019).

Alternatively, P2X7 activation may be important in the eradication of certain types of tumor. P2X7 activation has been demonstrated to induce apoptosis in acute myeloid cells but not haematopoietic stem cells (Salvestrini et al., 2017). A useful review of the literature is presented by Roger et al., where therapeutic strategies for solid tumors including promoting the cytolytic effect of ATP, are discussed (Roger et al., 2015). Many *in vitro* studies have shown that ATP or BzATP can be cytotoxic to tumor cells (Roger et al., 2015) and some have shown an effect of ATP on melanoma *in vivo* (White et al., 2009). Exploitation of the high level of expression of P2X7 on tumor cells to stimulate tumor cell death is an option explored by (De Andrade Mello et al., 2017). This study used hyperthermia to enhance membrane fluidity and potentiate ATP-induced cytotoxicity

via P2X7 in colon cancer cells *in vitro* (De Andrade Mello et al., 2017) although such an approach has not yet been tested *in vivo*. This does highlight the possibility of using positive allosteric modulators to provide a similar enhancement of P2X7-induced cell death.

Summarising therapeutic interventions, enhancement of P2X7 responses could be beneficial in infectious diseases (particularly with intracellular bacteria and parasites) to boost microbial defences, in anti-tumor immunity, and induction of tumor cell death. More research is required to develop selective PAMs and to determine their mechanisms of action both *in vitro* and *in vivo*.

POSITIVE ALLOSTERIC MODULATORS OF P2X7

A number of chemically distinct molecules have been suggested to act as positive modulators of P2X7. Clemastine, a first-generation anti-histamine, acts to positively modulate P2X7 in mouse and human macrophages (Norenberg et al., 2011). The combination of clemastine and ATP could enhance P2X7-mediated whole-cell currents, Ca^{2+} entry, pore-formation, and IL-1β release from human monocyte-derived macrophages and murine bone marrow-derived macrophages (Norenberg et al., 2011). Clemastine is thought to bind extracellularly to an allosteric site and concentration-response experiments using whole-cell recordings revealed an effect on sensitivity to agonist but not efficacy (Norenberg et al., 2011) therefore showing a Type II PAM effect (**Table 1**). There have been few studies so far investigating the effects of clemastine-induced potentiation of P2X7 in a biological setting. In a murine model of amyotrophic lateral sclerosis (ALS), a short treatment with clemastine (from postnatal day 40 to day 120) could delay the disease onset and extend the survival of SOD1-G93A mice by ~10% (Apolloni et al., 2016). Spinal microglia taken from these mice during the symptomatic phase highlighted that clemastine also stimulated autophagic flux and decreased SOD-1 levels. Whether or not this effect was P2X7-dependent was not investigated in this study, but clemastine treatment was observed to enhance the expression of both P2X7 and P2Y12 (Apolloni et al., 2016). A study by Su et al., investigated the effect of clemastine on chronic unpredictable mild stress and depressive-like behavior in BALB/c mice (Su et al., 2018). Clemastine could limit IL-1β and TNF-α production in the hippocampus, suppress microglial M1-like activation, and improve astrocytic loss within the hippocampus (Su et al., 2018). They also show that clemastine treatment resulted in downregulation of hippocampal P2X7 expression (Su et al., 2018). However, whether these effects of clemastine were P2X7-dependent was not investigated.

Isatin (1*H*-ondole-2,3-dione) is found within plant and animal tissues, including human tissues (concentrations range from <0.1 to 10 μM). Several isatin derivatives exist with a diverse array of properties (anti-microbial, anti-convulsant, anti-inflammatory, and anti-cancer) and biological targets (proteases, kinases, and caspases). *N*-alkylated isatin derivatives which typically bind to tubulin to destabilise microtubules, were identified to enhance IL-1β secretion in a P2X7-dependent manner (Sluyter and Vine, 2016). In contrast to isatin or the parent synthetic molecule, 5, 7-dibromoisatin, the derivatives 5, 7-dibromo-*N*-(*p*-methoxybenzyl) isatin (NAI), and 3-4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidenamino] phenylpropanoic acid (NAI-imine) could enhance P2X7-induced IL-1β release from J774 mouse macrophages (Sluyter and Vine, 2016). However, neither NAI or NAI-imine potentiated ATP-induced responses including dye uptake and cell death suggesting that these chemicals may act downstream of the P2X7 receptor (Sluyter and Vine, 2016). Without further experimental evidence for their mechanism of activation, we have not classified the isatin derivatives as PAMs of P2X7.

Ivermectin, as previously discussed, is a commonly utilized PAM for P2X4. Challenging the selectivity of ivermectin for P2X4 within the family, Norenberg et al., demonstrated that ivermectin potentiated human P2X7 receptors but not murine P2X7 (Nörenberg et al., 2012). Utilizing electrophysiological and fluorometric methods, they observed potentiation of ATP-induced currents and Ca^{2+} influx in cells expressing human P2X7, but not rat or mouse P2X7 (Nörenberg et al., 2012). Notably, ivermectin could not potentiate other P2X7-driven functions such as YO-PRO-1 dye uptake (Nörenberg et al., 2012). Concentration-response experiments reveal that ivermectin has a minor effect on the EC_{50} value for ATP and can increase the maximum response (Nörenberg et al., 2012) suggesting classification as a mixed Type I/II PAM effect (**Table 1**). Ivermectin has been suggested to drive P2X4/P2X7/Pannexin-1 signaling to enhance numerous cell death pathways including apoptosis, necrosis, pyroptosis, and autophagy in cancer cells (Draganov et al., 2015).

Ginsenosides are steroid-like glycosides that are predominantly obtained from the roots of the plant genus *Panax ginseng*. Our lab first described four protopanaxadiol ginsenosides [Rb1, Rh2, Rd, and the metabolite compound K (CK)] that could potentiate ATP-activated P2X7 currents, dye uptake, and intracellular Ca^{2+} concentrations, with the most potent ginsenoside CK enhancing cell death toward a non-lethal concentration of ATP (Helliwell et al., 2015). Using molecular modeling and computational docking, we identified a novel binding site in the central vestibule region of human P2X7 (Bidula et al., 2019b) shared by other P2X receptors such as P2X4 (Dhuna et al., 2019). This predicted allosteric site involves amino acid residues Ser60, Asp318 and Leu320 in the β-strands connecting the orthosteric binding site to the transmembrane domains (Bidula et al., 2019b). This region is intimately involved in gating and more work now needs to be done to explore the mechanism of potentiation. Recently, we explored the effect of ginsenosides on P2X7-dependent cell death. High ATP (3 mM) was shown to induce an unregulated form of cell death in J774 mouse macrophages, while conversely, potentiation of a non-lethal concentration of ATP by ginsenoside CK could enhance apoptotic cell death in a caspase-dependent manner (Bidula et al., 2019a). In contrast to high ATP, the effect of ginsenoside CK could be reversed *via* the chelation of extracellular Ca^{2+},

scavenging mitochondrial ROS, Bax inhibition, or by caspase inhibitors suggesting that different intracellular signaling events were involved following positive modulation (Bidula et al., 2019a).

Tenidap, a COX/5-LOX inhibitor and anti-inflammatory drug was discovered to be a potentiator of mouse P2X7 enhancing ATP-mediated cytotoxicity and Lucifer yellow dye uptake (Sanz et al., 1998). From the dose-response experiments performed in an LDH release assay (Sanz et al., 1998), it appears tenidap has mixed Type I/II PAM effects at P2X7 (**Table 1**). It is not known whether the effect of tenidap is restricted to mouse P2X7; no studies on human P2X7 can be found.

Polymyxin B, an antibiotic with bactericidal action against almost all Gram-negative bacteria, was identified to have potentiating action at P2X7 enhancing Ca^{2+} influx, membrane permeabilization, and cytotoxicity to low agonist concentrations (Ferrari et al., 2004). Interestingly, treatment with the irreversible inhibitor oxidised ATP or genetic ablation of P2X7 rendered cells insensitive to the synergistic effects of ATP and polymyxin B, but this effect was not replicated by the reversible P2X7 inhibitor KN-62 (Ferrari et al., 2004). Polymyxin B appears to left-shift the ATP concentration-response curve and increase the maximum response (Ferrari et al., 2004) thus it has mixed Type I/II PAM effects (**Table 1**). Polymyxin B nonapeptide, a derivative of polymyxin B lacking the N-terminal fatty amino acid 6-methylheptanoic/octanoic-Dab residue, did not have the same activity at P2X7 (Ferrari et al., 2007).

Agelasine and garcinoloic acid are two natural products capable of potentiating P2X7. Agelasines are bioactive 7,9-dialkylpurinium salts isolated from a marine sponge, whereas garcinolic acid is a xanthone derived from flowering plants of the species *Garsinia* (Fischer et al., 2014). Both agelasine and garcinolic acid compounds could potentiate P2X7 responses in HEK-293, A375 melanoma, and mouse microglial cells, but only garcinolic acid could significantly enhance P2X7-induced dye uptake (Fischer et al., 2014). Information regarding the type of PAM effect could not be extracted from the study as the effect of agelasine/garcinolic acid on the ATP concentration-response curves were not reported.

Other positive modulators of P2X7 include GW791343 (2-[(3,4- Difluorophenyl) amino]-*N*-[2-methyl-5-(1-piperazinylmethyl) phenyl]-acetamide trihydrochloride) a negative modulator at human P2X7, but a positive modulator at rat P2X7 (Michel et al., 2008a). Using ethidium uptake experiments to measure P2X7 responses, GW791343 increases potency and efficacy of the agonist BzATP at rat P2X7 expressed in HEK-293 cells (Michel et al., 2008a) suggesting a mixed Type I/II PAM effect (**Table 1**). Key structural differences exist between different species of P2X7 receptor and amino acid residue at position 95 is thought to be involved in coordinating GW791343 (Michel et al., 2008b). Anaesthetics such as ketamine, propofol, thiopental and sevofluranehave been identified as positive modulators of P2X7 in two independent studies (Nakanishi et al., 2007; Jin et al., 2013). Various phospholipids such as lysophosphatidylcholine, sphingophosphorylcholine, and hexadecylphosphorylcholine, can modulate the potency of ATP towards P2X7 (Michel and Fonfria, 2007). When used at sub-cytotoxic concentrations, each of these lipids could potentiate ethidium accumulation and P2X7-dependent IL-1β production from cells expressing recombinant or endogenous P2X7 respectively. However, when used at higher concentrations, the lipids induce an increase in intracellular Ca^{2+}, radioligand binding, and cytotoxicity (Michel and Fonfria, 2007). Therefore, it is unclear whether the lipids are having a direct effect at P2X7 or simply inducing changes in the properties of the membrane itself. Phosphoinositides (anionic signaling phospholipids) can also positively modulate P2X7 *via* short, semi-conserved polybasic domain located in the proximal C-terminus of P2X subunits (Bernier et al., 2013). A single study has identified that P2X7 can be allosterically modulated by the glycosaminoglycan chains of CD44 proteoglycans present on Chinese hamster ovary (CHO) cells (Moura et al., 2015). The presence of these GAGs on the cell surface significantly increased the sensitivity of cells to ATP, potentiating Ca^{2+} influx and pore formation (Moura et al., 2015). Moreover, cells defective in GAG biosynthesis were protected from P2X7-dependent cell death (Moura et al., 2015). These works open up the possibility that allosteric modulation of P2X7 could occur *in vivo via* a multitude of mechanisms.

As the known number of positive allosteric modulators for P2X7 begins to increase, the question arises as to where positive modulation of P2X7 would be therapeutically beneficial. It has been well documented that P2X7 plays pivotal roles in immunity to infection and loss-of-function SNPs in P2X7 have proven deleterious. P2X7 has been demonstrated to provide immune protection towards viral (dengue), bacterial (chlamydia, periodontitis, tuberculosis), fungal (paracoccidioidomycosis), parasitic (leishmaniasis, trypanosomiasis, toxoplasmosis, amoebiasis, malaria), and helminth (schistosomiasis) infections. The causative agents of these diseases directly impact billions of people of worldwide and indirectly put many others at risk. Thus, the identification of novel positive allosteric modulators of P2X7 and further exploration into their biological effects would be significantly beneficial in the development of novel treatments to boost immune defences.

With the identification that positive modulation by ginsenoside CK could calibrate cell death responses of macrophages, promoting apoptotic cell pathways over lytic cell death pathways (Bidula et al., 2019a), we hypothesize that the ability to be able to pharmacologically promote these types of cell death pathways could be beneficial in the removal of pathogens, particularly intracellular pathogens such as mycobacteria and parasites which are not always effectively recognised by the immune system. Due to the role of P2X7 in regulating several cell death pathways, further investigation into whether other positive modulators could selectively promote alternative cell death pathways involved in the removal of pathogens could be important in the resolution of the infections listed above.

Another area in which positive modulation could be beneficial is in the treatment of cancers. P2X7 appears to participate in ameliorating myeloma, glioblastoma, non-small cell lung carcinoma, and melanoma, but the studies concerning cancer are often contradictory. However, a common characteristic among cancer cells is that many of them exhibit higher

expression of P2X7 and that ATP at the tumor site is often abundant (Pellegatti et al., 2008; Roger et al., 2015). Stimulation of numerous cancer cell lines with high concentrations of ATP *in vitro* results in decreased viability of these cells. In cancer patients, it may be plausible to try and target cancer cells in two ways: administering a positive modulator to amplify the effects of enhanced local ATP concentrations around the tumor, activating P2X7 and inducing cell death or, alternatively, the use of an antibody-drug conjugate to target P2X7 specifically on these cells could be employed. Attaching a positive modulator to an antibody specific to P2X7, especially when targeting tumors with enhanced expression of P2X7, could deliver the modulator to where it is needed, amplify P2X7 responses on these cells, and induce death of the cancer cells. An issue arising from this method however, is if the cancer patient has any underlying pathologies associated with enhanced expression of P2X7, then targeting this receptor through antibody-drug conjugates might result in off-target effects and death of healthy cells.

CONCLUSIONS

Collectively it appears that there is good evidence that positive allosteric modulation of P2X2, P2X4, and P2X7 receptors may be of therapeutic benefit in a number of different conditions summarised in **Figure 2**. It is also clear that there may be endogenous molecules particularly in the central nervous system, that could act as positive modulators to enhance the action of the physiological agonist ATP (e.g., neurosteroids on P2X2 and P2X4). With regard to the question posed in the title, to inhibit or enhance, we have tried to present a balanced view of the knowledge surrounding the major physiological and pathophysiological roles for P2X receptors. There is a strong case for inhibition of several P2X receptors in a variety of diseases and clinical development of candidate compounds is in progress. However, this does not exclude the development of positive modulators for use in other disorders. We hope that we have highlighted these opportunities. Similar to other ligand-gated ion channels (NMDA receptors, nAchR) one challenge lies in drug selectivity for different forms of ion channels, typically subunit composition. With this in mind, knowing more about pharmacology of splice variants and polymorphic variants may be important for homomeric P2X receptors and understanding the differential pharmacology of heteromeric P2X receptors. With the advances in structural information and continued progress in allosteric binding pocket identification, plus access to the relevant animal models of disease, positive modulation of P2X receptors may become a fruitful area of research.

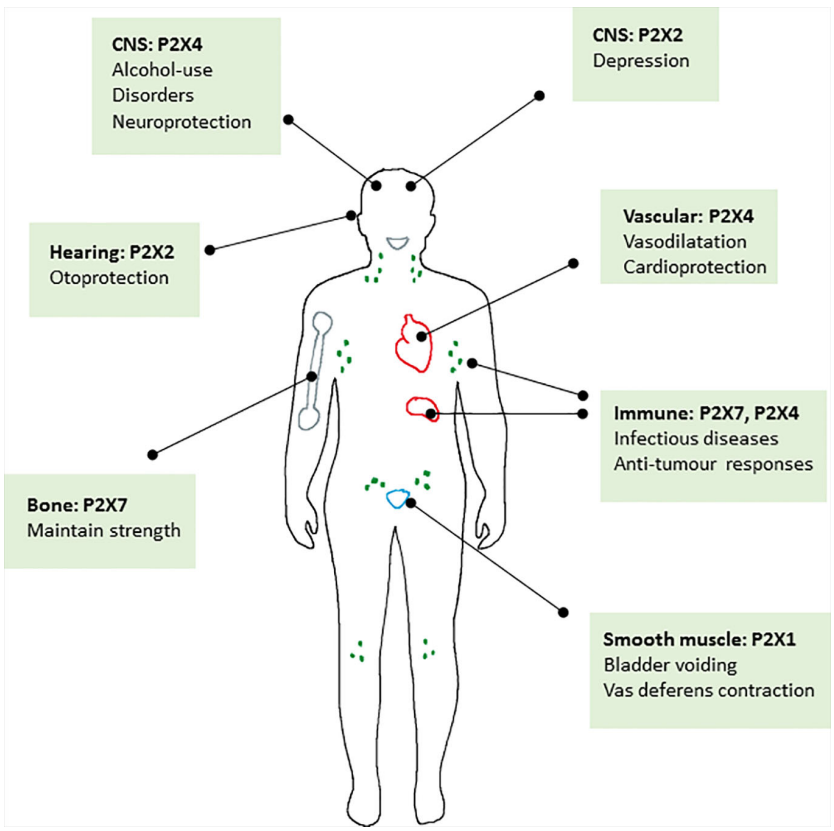

FIGURE 2 | Schematic diagram summarising the major roles of P2X receptors in the body where positive allosteric modulators may have a therapeutic benefit.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the review. LS compiled and edited the final version.

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