

## Sustained Calcium Entry through P2X Nucleotide Receptor Channels in Human Airway Epithelial Cells\*

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**Purinergic receptor stimulation has potential therapeutic effects for cystic fibrosis (CF). Thus, we explored roles for P2Y and P2X receptors in stably increasing  $[Ca^{2+}]_i$  in human CF (IB3-1) and non-CF (16HBE14o<sup>-</sup>) airway epithelial cells. Cytosolic  $Ca^{2+}$  was measured by fluorospectrometry using the fluorescent dye Fura-2/AM. Expression of P2X receptor (P2XR) subtypes was assessed by immunoblotting and biotinylation. In IB3-1 cells, ATP and other P2Y agonists caused only a transient increase in  $[Ca^{2+}]_i$  derived from intracellular stores in a  $Na^+$ -rich environment. In contrast, ATP induced an increase in  $[Ca^{2+}]_i$  that had transient and sustained components in a  $Na^+$ -free medium; the sustained plateau was potentiated by zinc or increasing extracellular pH. Benzoyl-benzoyl-ATP, a P2XR-selective agonist, increased  $[Ca^{2+}]_i$  only in  $Na^+$ -free medium, suggesting competition between  $Na^+$  and  $Ca^{2+}$  through P2XRs. Biochemical evidence showed that the P2X<sub>4</sub> receptor is the major subtype shared by these airway epithelial cells. A role for store-operated  $Ca^{2+}$  channels, voltage-dependent  $Ca^{2+}$  channels, or  $Na^+/Ca^{2+}$  exchanger in the ATP-induced sustained  $Ca^{2+}$  signal was ruled out. In conclusion, these data show that epithelial P2X<sub>4</sub> receptors serve as ATP-gated calcium entry channels that induce a sustained increase in  $[Ca^{2+}]_i$ . In airway epithelia, a P2XR-mediated  $Ca^{2+}$  signal may have therapeutic benefit for CF.**

In cystic fibrosis (CF),<sup>1</sup> cyclic AMP- and protein kinase A-dependent transepithelial  $Cl^-$  transport is impaired because of

mutations in the CF gene that encodes for the protein, the “cystic fibrosis transmembrane conductance regulator” or CFTR (1). Originally, CFTR was thought to function exclusively as a low conductance  $Cl^-$  channel (2, 3). More recently, it has become clear that CFTR also regulates a series of other transporters and ion channels, such as the  $Cl^-/HCO_3^-$  exchanger, the  $Na^+:HCO_3^-$  cotransporter, epithelial  $Na^+$  channels,  $K^+$  channels, and aquaporin water channels (4, 5). Although the exact mechanisms of the regulation of these proteins by CFTR are not yet fully understood, it is clear that impaired  $Cl^-$  transport is shared as a key disease phenotype by CF epithelia from all affected tissues and that this pathway is lost in CF. Therefore, activation of a cAMP-independent  $Cl^-$  secretory pathway through exploitation of a naturally expressed epithelial protein could be of interest for CF therapy. In certain cases, stimulation of  $Ca^{2+}$ -dependent  $Cl^-$  channels can correct the impaired  $HCO_3^-$  secretion in CF cells (6, 7).

It is widely accepted that CFTR plays a crucial role in ATP release from cells (8–10). The same is true for *mdr* ABC transporters in hepatocytes and heterologous cells (11, 12). Once ATP is released into the extracellular space, it can bind to purinoceptors regulating a variety of functions in different epithelia (13–15). ATP and other agonists of purinoceptors are known to increase intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) potentially in airway epithelial cells which, in turn, leads to stimulation of  $Cl^-$  secretion (14–17) and inhibition of  $Na^+$  absorption (18–22). In fact, earlier studies have proposed the use of UTP and non-hydrolyzable UTP analogs as therapeutic agonists targeted to the P2Y<sub>2</sub> receptors in the treatment of CF lung disease (23, 24).

Purinoceptors are divided into two classes: P1 or adenosine receptors, and P2, which recognize primarily extracellular ATP, ADP, UTP, and UDP. The P2 receptors are further subdivided into two subclasses. P2X receptors are extracellular ATP-gated calcium-permeable non-selective cation channels that are modulated by extracellular  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $H^+$ , and metal ions such as  $Zn^{2+}$  and/or  $Cu^{2+}$  (25). P2Y receptors couple to heterotrimeric G proteins and phospholipases (primarily phospholipase C $\beta$ ) to raise intracellular free calcium concentration (26). In CF epithelial cells from multiple tissues, expression of P2X and P2Y receptors appears unaffected, offering the possibility to increase  $[Ca^{2+}]_i$  through targeting a naturally expressed receptor in the apical or basolateral membrane domains (27, 28). Nonetheless, in different CF epithelial cell models, the desensitization of P2Y receptors and the transient nature of the  $Ca^{2+}$  response upon chronic and repeated delivery of a P2Y-specific agonist have made it difficult to generate stable stimuli for ion secretion (7, 29).

In this study, we used both CF (IB3-1) (30) and non-CF (16HBE14o<sup>-</sup>) (31) human airway epithelial cell models, to dissect out P2X-specific and P2Y-specific mechanisms of trigger-

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<sup>1</sup> The abbreviations used are: CF, cystic fibrosis; ASL, airway surface liquid; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; NMDG, N-methyl-D-glucamine; P2XR, P2X purinergic receptor channel; SOC, store-operated calcium channel; TRP, transient receptor potential channel; PBS, phosphate-buffered saline; ADP $\beta$ S, adenosine 5'-[ $\beta$ -thioldiphosphate; 2MeSATP, 2-methylthio ATP;  $\alpha,\beta$ -meATP, methylene ATP; BzBzATP, benzoyl-benzoyl-ATP; 2APB, 2-amino-ethoxyphenyl borate.

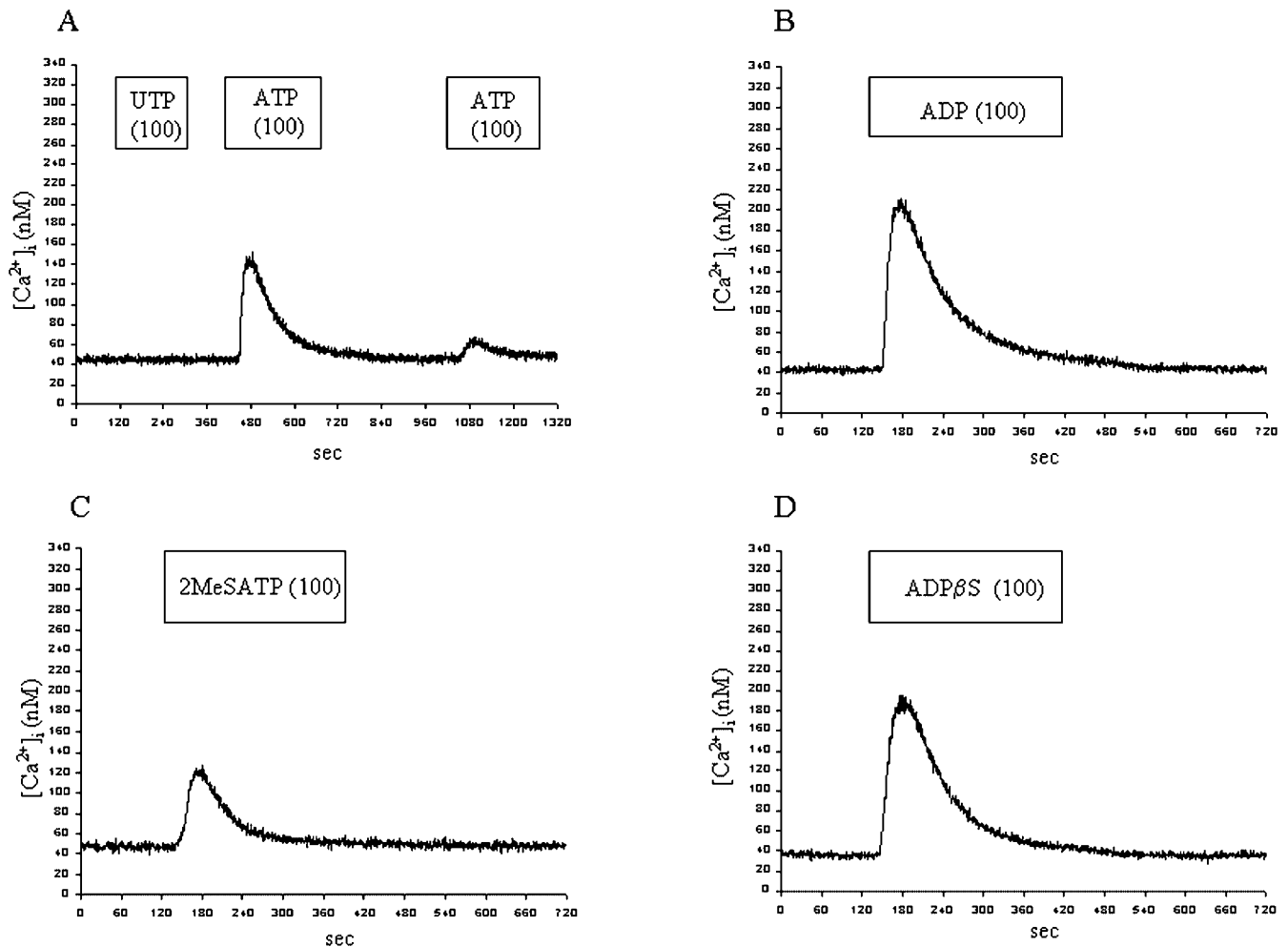


FIG. 1. Original traces showing the effects of ATP and UTP (100  $\mu\text{M}$  each) (A), ADP (100  $\mu\text{M}$ ) (B), 2MeSATP (100  $\mu\text{M}$ ) (C), and ADP $\beta$ S (100  $\mu\text{M}$ ) (D) on  $[\text{Ca}^{2+}]_i$ . IB3-1 cells were superfused with  $\text{Na}^+$ -containing medium (solution A). A, please note that the second application of ATP was without effect. In these traces and in all others below, please note that there is a time lag of 10–15 s before agonist-containing perfusate enters the cuvette. As all of these experiments were performed on coverslips prepared on the same day, a calibration was used on the same cell preparation to allow conversion and plotting of the data as cytosolic calcium.

ing an increase in  $[\text{Ca}^{2+}]_i$ . We characterized a broad range of P2Y-selective, P2X-selective, and non-discriminant P2Y and P2X agonists under different chemical and ionic conditions to explore possible strategies to elicit an increase in  $[\text{Ca}^{2+}]_i$  that is sustained and prolonged. Results described herein, using Fura-2/AM-based imaging, show that activation of P2Y and P2X receptors increases  $[\text{Ca}^{2+}]_i$  by completely distinct mechanisms. P2Y receptors elicit a transient increase in  $[\text{Ca}^{2+}]_i$  derived from intracellular endoplasmic reticulum (ER) stores, whereas P2X receptors trigger a sustained rise in  $[\text{Ca}^{2+}]_i$ , allowing  $\text{Ca}^{2+}$  influx from the extracellular space. In addition, biochemical evidence shows that the P2X<sub>4</sub> receptor is the major epithelial subtype present in both cell lines. Thus, we conclude that epithelial P2X receptors function as ATP-gated  $\text{Ca}^{2+}$  entry channels in the plasma membrane and have profound potential as a target for CF pharmacotherapy.

#### MATERIALS AND METHODS

**Cell Cultures**—IB3-1 cells derive from airway epithelia of a CF patient carrying two different mutations of the CFTR gene, the most common trafficking mutation ( $\Delta\text{Phe-508}$ ) and a premature stop codon mutation (W1282X) (30). 16HBE14o<sup>-</sup> cells are non-CF or normal airway epithelial cells, which express CFTR at the plasma membrane. The cells were grown on Vitrogen 100-coated tissue-culture flasks in 5%  $\text{CO}_2$  incubator at 37 °C. IB3-1 cells were cultured in LHC-8 (Biofluids, Rockville, MD) medium supplemented with 5% fetal bovine serum (Invitrogen), 100 units/ml penicillin/streptomycin (Invitrogen), 1  $\times$  L-glutamine

(Invitrogen), and 1.25  $\mu\text{g}/\text{ml}$  Fungizone (Invitrogen). 16HBE14o<sup>-</sup> cells were cultured in minimum Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. When cells reached confluency, they were washed twice with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS. The cells were then suspended using trypsin/EDTA solution and plated on diluted Vitrogen-coated (collagen types I and IV diluted 1:15 in Dulbecco's phosphate-buffered saline) glass coverslips. For  $[\text{Ca}^{2+}]_i$  measurements, cells were used 48–72 h after plating.

**Fura-2 Imaging of Intracellular  $\text{Ca}^{2+}$** —Cytosolic  $\text{Ca}^{2+}$  concentration was measured with dual excitation wavelength fluorescence microscopy (Deltascan, Photon Technologies, Princeton, NJ) after cells were loaded with the permeant form of the fluorescence dye Fura-2/acetoxymethyl ester (Fura-2/AM; Teflabs, Austin, TX). Fura-2 fluorescence was measured at an emission wavelength of 510 nm in response to the excitation wavelength of 340 and 380 nm, alternated at a rate of 50 Hz by a computer-controlled chopper assembly. Ratios (340/380 nm) were calculated at a rate of 5 points/s using PTI software. Cells were incubated in Dulbecco's phosphate-buffered saline containing 2 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$  in the presence of 5  $\mu\text{M}$  Fura-2/AM and 1 mg/ml Pluronic F-127 dissolved in  $\text{Me}_2\text{SO}$  for 120 min to allow loading of the dye into the cells. After loading, coverslips were rinsed at least for 10 min in Dulbecco's phosphate-buffered saline to remove extracellular Fura-2/AM and the surfactant and were positioned in the cuvette at a 45° angle from the excitation light. Two glass capillary tubes were inserted into the top of the cuvette out of the patch of the excitation light. One tube was extended to the bottom of the cuvette and connected by way of polyethylene tubing to an infusion pump. The other capillary tube was positioned at the top of the cuvette and served to remove fluid from the cuvette. The volume of the cuvette was  $\sim 1.5$  ml, and the flow rate was

~5 ml/min. It is important to note that switch in perfusion solutions is removed in time and space for the cuvette, such that a 10–15-s time lag exists before agonist is exposed to the cells. Experiments were performed at room temperature. Fluorescence intensities at both wavelengths were assessed, and only those preparations in which there were >200,000 counts/s for both wavelengths were used for experiments. At the beginning of each experiment, cells were perfused with solution A (see below), and the fluorescence ratio was monitored for at least for 100 s to establish a stable base-line value. Agonists and antagonists were then added to the appropriate solutions (see later). The 340/380 nm ratios ( $R$ ) were converted into  $[Ca^{2+}]_i$  values using the equations of Grynkiewicz *et al.* (32) as follows:  $[Ca^{2+}]_i = K_d \times ((R - R_{min})/(R_{max} - R)) \times (S_{380}/S_{340})$  where  $K_d$  is the dissociation constant of Fura-2 for

$Ca^{2+}$ ,  $R_{max}$  and  $R_{min}$  are  $R$  values under saturating and  $Ca^{2+}$ -free conditions, respectively, and  $S_{380}$  and  $S_{340}$  are the fluorescent signals ( $S$ ) emitted by  $Ca^{2+}$ -free ( $f$ ) and  $Ca^{2+}$ -bound ( $b$ ) forms of Fura-2 at a wavelength of 380 nm. *In situ* cell calibrations were accomplished after the cells were permeabilized with ionomycin (2  $\mu$ M) under  $Ca^{2+}$ -free (10 mM EGTA) and saturating  $Ca^{2+}$  (3 mM  $CaCl_2$ ) conditions. The  $K_d$  was assumed to be 224 nM (32).

**Fura-2 Quenching Experiments**—Cells were loaded and washed as described for intracellular  $[Ca^{2+}]$  measurement. Fluorescence signal was measured at 359 nm (isobestic wavelength) in the presence of  $MnCl_2$  (500  $\mu$ M) to detect  $Ca^{2+}$ -independent changes in Fura-2 fluorescence (33).

**Immunoblotting with P2X Receptor Channel Isoform-specific Antibodies**—Cells were lysed in a buffer containing 10 mM Tris, 0.5 mM NaCl, 0.5% Triton X-100, 50  $\mu$ g/ml aprotinin (Sigma), 100  $\mu$ g/ml leupeptin (Sigma), and 100  $\mu$ g/ml pepstatin A (Sigma) adjusted to pH 7.2–7.4. Twenty micrograms of protein were run per lane and separated on an 8% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Osmonics, Westborough, MA). Immunoblotting was performed with a rabbit polyclonal antibody to P2X<sub>4</sub> (Alomone Laboratories, Jerusalem, Israel) at a dilution of 1:500. P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>7</sub> antibodies were also obtained from Alomone Laboratories and were tested in a similar manner. Reactivity was detected by horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:3,000 dilution, New England Biolabs, Beverly, MA). Enhanced chemiluminescence was used to visualize the secondary antibody.

**Biotinylation of Plasma Membrane P2X Receptor Channels**—Cells were seeded on Vitrogen-coated (collagen types I and IV diluted 1:15 in Dulbecco's phosphate-buffered saline) 12-mm filters and grown as polarized monolayers with a transepithelial resistance that exceeded 400 ohms/cm<sup>2</sup>. Cells were placed on ice and washed 3 times with cold PBS supplemented with 0.1 mM  $CaCl_2$  and 1.0 mM  $MgCl_2$ . Cells were then incubated in 1.0 mg/ml poly(ethylene)oxid maleimide (Pierce) or sulfo-NHS-LC biotin (Pierce) in cold supplemented PBS for 25 min at 4 °C. Cells were washed 4 times with cold supplemented PBS, and the biotin was quenched with 0.1% bovine serum albumin (Sigma). Cells were then washed 3 times with cold supplemented PBS. Alternatively, cells could be biotinylated with biocytin hydrazide. Filters were first incubated in 300  $\mu$ l of a stock solution containing 30 mM  $NaIO_4$  and 600  $\mu$ l of a stock solution containing 100 mM sodium acetate and 0.02% sodium azide, pH 5.5, for 30 min at room temperature in the dark. Filters were washed and subsequently incubated with 1.0 mg/ml biocytin hydrazide

TABLE I  
Maximum changes in Fura-2 fluorescence in IB3-1 cells in  $Na^+$ -containing medium

$\Delta$ ratios (340/380 nm) are maximum changes in Fura-2 fluorescence in response to purinergic agonists *versus* basal fluorescence. Values for % are percent changes in fluorescence *versus* ATP (100  $\mu$ M), except ADP (100  $\mu$ M) +  $Ca^{2+}$  free media whose value for % is *versus* ADP (100  $\mu$ M). Values are means  $\pm$  S.D.;  $n$  = number of experiments.

	$\Delta$ ratio	%	$n$
ATP (100 $\mu$ M)	0.30 $\pm$ 0.11	100	15
ATP (10 $\mu$ M)	0.20 $\pm$ 0.10	67	4
ATP (100 $\mu$ M) + suramin (100 $\mu$ M)	0.04 $\pm$ 0.02 <sup>a</sup>	13	4
ATP (100 $\mu$ M) + $Ca^{2+}$ -free media	0.18 $\pm$ 0.03 <sup>a</sup>	60	5
ADP (100 $\mu$ M)	0.38 $\pm$ 0.17	127	8
ADP (100 $\mu$ M) + $Ca^{2+}$ -free media	0.16 $\pm$ 0.05 <sup>b</sup>	42	4
ADP $\beta$ S (100 $\mu$ M)	0.37 $\pm$ 0.06	123	7
ADP $\beta$ S (10 $\mu$ M)	0.28 $\pm$ 0.07	93	3
2MeSATP (100 $\mu$ M)	0.24 $\pm$ 0.06	80	3
ATP (100 $\mu$ M) + $ZnCl_2$ (20 $\mu$ M)	0.25 $\pm$ 0.07	83	4
ATP (100 $\mu$ M) at $pH_e = 7.9$	0.38 $\pm$ 0.18	127	2
ATP (100 $\mu$ M) at $pH_e = 6.4$	0.22 $\pm$ 0.02	73	2
UTP (100 $\mu$ M)	No increase	0	6
UDP (100 $\mu$ M)	No increase	0	3
Adenosine (100 $\mu$ M)	No increase	0	4
BzBzATP (100 $\mu$ M)	No increase	0	3
$\alpha, \beta$ -MeATP (100 $\mu$ M)	No increase	0	3

<sup>a</sup>  $p < 0.05$  relative to ATP (100  $\mu$ M).

<sup>b</sup>  $p < 0.05$  relative to ADP (100  $\mu$ M).

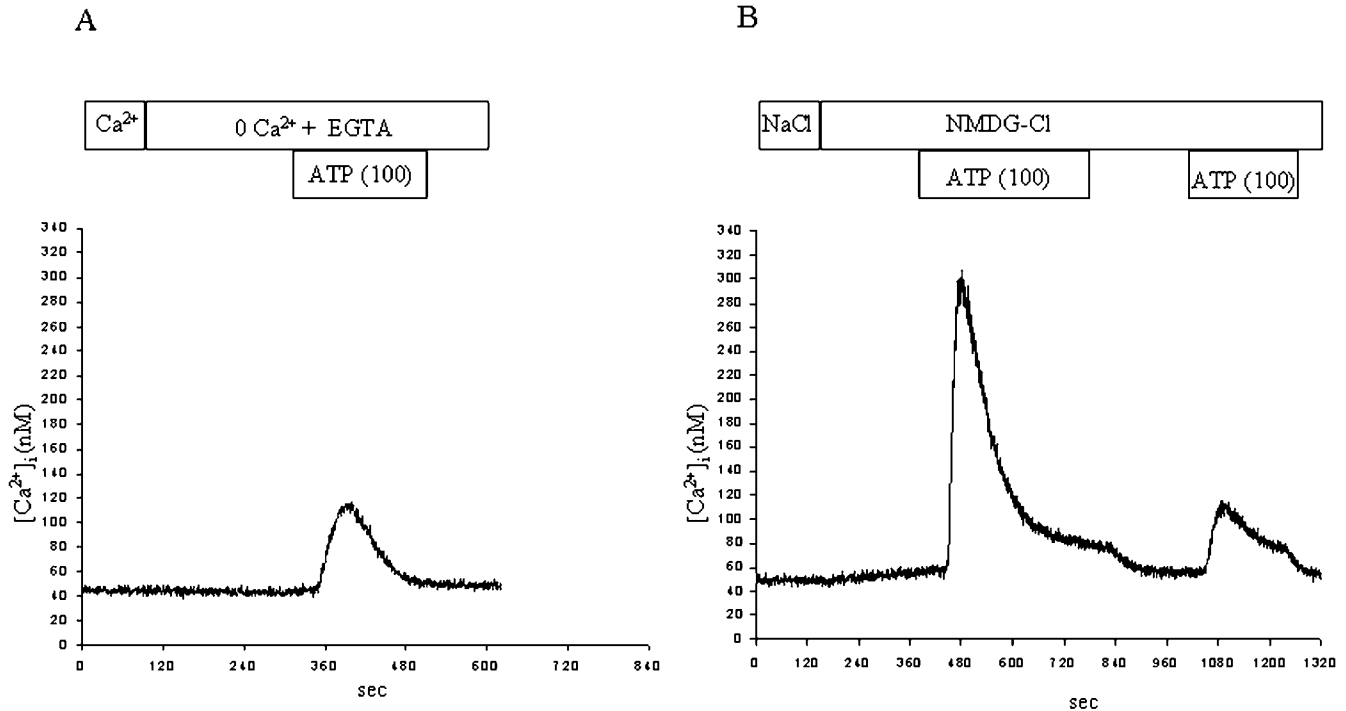


FIG. 2. Original traces showing the effects of ATP (100  $\mu$ M) on  $[Ca^{2+}]_i$  in IB3-1 cells exposed to nominally  $Ca^{2+}$ -free,  $Na^+$ -containing solution (A) and in cells exposed to  $Ca^{2+}$ -containing  $Na^+$ -free solution (B) as indicated. B, please note the slight sustained increase in  $[Ca^{2+}]_i$  upon substitution of  $Na^+$  by NMDG. This sustained plateau was the first hint that in  $Na^+$ -free medium  $Ca^{2+}$  entry channels could also be involved in the ATP-induced sustained  $Ca^{2+}$  response.



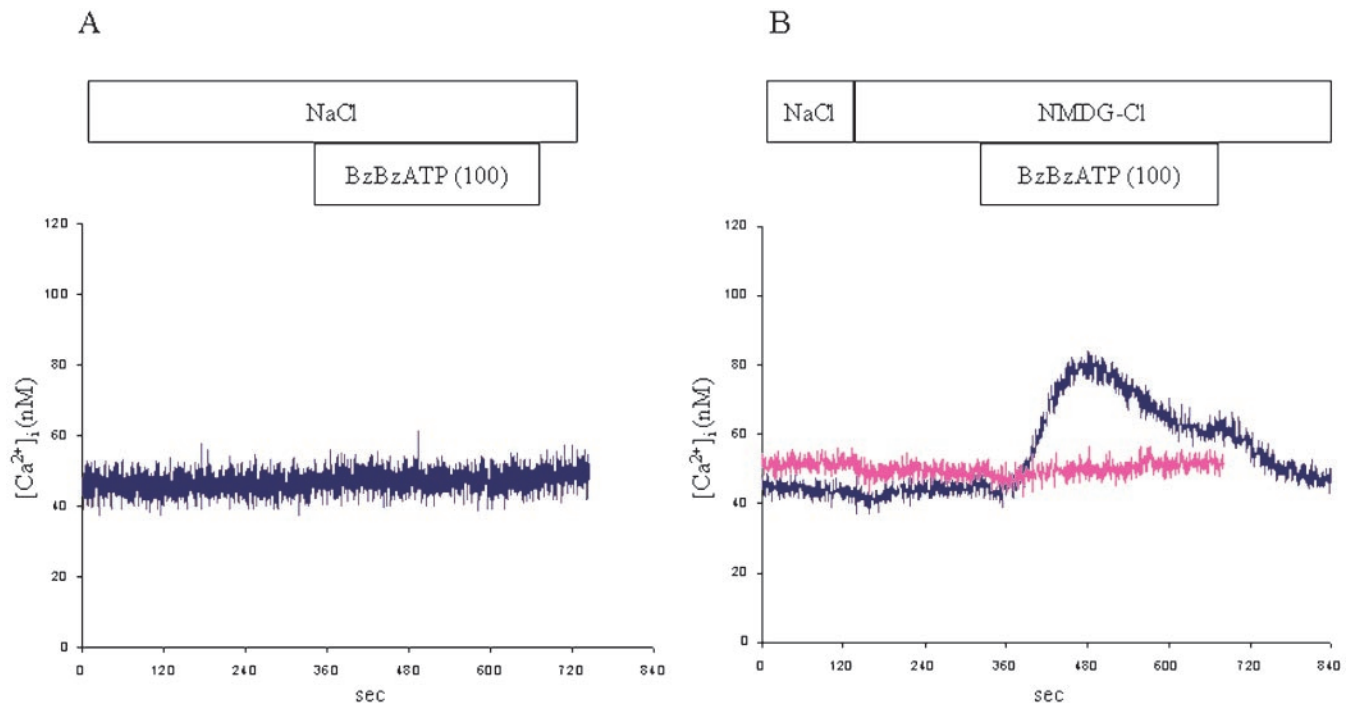


FIG. 3. *A* includes an original trace showing the lack of an effect of BzBzATP (100  $\mu$ M) on  $[Ca^{2+}]_i$  in IB3-1 cells in  $Na^+$ -containing medium. *B* shows an original trace where cells exposed to  $Na^+$ -free medium are responsive to BzBzATP (100  $\mu$ M) with a rise in  $[Ca^{2+}]_i$  in IB3-1 cells. Note that a second trace (in magenta) is shown to illustrate the lack of effect of BzBzATP (100  $\mu$ M) in a  $Ca^{2+}$ -free and  $Na^+$ -free solution.

TABLE II  
Maximum changes in Fura-2 fluorescence in IB3-1 cells in  $Na^+$ -free medium

$\Delta$ ratios (340/380 nm) are maximum changes in Fura-2 fluorescence in response to purinergic agonists *versus* basal fluorescence. All values for % are percent changes in fluorescence *versus* ATP (100  $\mu$ M). Values are means  $\pm$  S.D.;  $n$  = number of experiments.

	$\Delta$ ratio	%	$n$
ATP (100 $\mu$ M)	$0.82 \pm 0.24^a$	100	11
ATP (100 $\mu$ M) + $Ca^{2+}$ -free media	$0.25 \pm 0.14^b$	31	4
ATP (100 $\mu$ M) + $ZnCl_2$ (20 $\mu$ M)	$0.77 \pm 0.21$	94	5
ATP (100 $\mu$ M) at $pH_e = 7.9$	$0.78 \pm 0.22$	95	5
ATP (100 $\mu$ M) at $pH_e = 6.4$	$0.22 \pm 0.06^b$	27	5
ATP (100 $\mu$ M) + KB-R7943 (30 $\mu$ M)	$0.70 \pm 0.13$	85	3
ATP (100 $\mu$ M) + high $[KCl]_e$ (40 mM)	$0.38 \pm 0.08^b$	46	4
ADP $\beta$ S (100 $\mu$ M)	$0.80 \pm 0.23^c$	98	3
BzBzATP (100 $\mu$ M)	$0.15 \pm 0.02^d$	18	5
BzBzATP (100 $\mu$ M) + $Ca^{2+}$ -free media	No increase	0	3
$\alpha,\beta$ -MeATP (100 $\mu$ M)	No increase	0	3

<sup>a</sup>  $p < 0.05$  relative to ATP (100  $\mu$ M) in sodium-containing medium (see Table I).

<sup>b</sup>  $p < 0.05$  relative to ATP (100  $\mu$ M) alone.

<sup>c</sup>  $p < 0.05$  relative to ADP $\beta$ S (100  $\mu$ M) in sodium-containing medium (see Table I).

<sup>d</sup>  $p < 0.05$  relative to BzBzATP (100  $\mu$ M) in sodium-containing medium (see Table I).

(Pierce) for 1 h at 4  $^{\circ}C$ . The reaction was quenched with 0.1 M Tris, pH 7.5. Cell lysates were collected as described above in immunoblotting procedures. Immobilized streptavidin beads (Pierce) were added to the lysates at a 1:10 dilution and rocked overnight at 4  $^{\circ}C$ . Beads were washed 3 times with lysis buffer and incubated in sample buffer for 5 min at 95  $^{\circ}C$ . The mixture was centrifuged, and the supernatant was loaded onto an SDS-PAGE gel. The immunoblotting procedure then continued as described above.

**Solutions**—Buffers for  $[Ca^{2+}]_i$  measurement contained (mmol/liter) the following: for solution A: NaCl 140, KCl 3,  $KH_2PO_4$  1.3,  $Na_2HPO_4$  8,  $MgCl_2$  1,  $CaCl_2$  2; for solution B: NaCl 140, KCl 3,  $KH_2PO_4$  1.3,  $Na_2HPO_4$  8,  $MgCl_2$  1, Na-EGTA 1; for solution C: NMDG-Cl 140, KCl 4.5, Hepes 10,  $MgCl_2$  1,  $CaCl_2$  2; and for solution D: NMDG-Cl 100, KCl 40, Hepes 10,  $MgCl_2$  1,  $CaCl_2$  2. The solutions are at pH 7.3 unless indicated otherwise. In Fura-2 quenching experiments  $MnCl_2$  (500  $\mu$ M) was added to  $Ca^{2+}$ - and EGTA-free solutions.

**Data Analysis**—Data are expressed as mean  $\pm$  S.D. An unpaired Student's  $t$  test was used to compare the data in different experimental groups. Results were considered significant if  $p < 0.05$ . For original Fura-2 traces shown in the figures, data are graphed with calibrated cytosolic free calcium on the y axis, because data from an individual preparation of cells was accumulated for all of the experiments in that figure where a calibration was also performed. Because not all data were generated from cells of the same passage or where a calibration was not performed for every preparation, the data in tables are shown as ratiometric data.

## RESULTS

**Purinergic Agonists Trigger a Transient Increase in  $[Ca^{2+}]_i$  in the Presence of Extracellular  $Na^+$  in IB3-1 Cells**—To test for the presence of purinergic receptors in IB3-1 cells, we measured the cytosolic free  $Ca^{2+}$  concentration after stimulation with different agonists to both P2Y and P2X receptors in physiologic bath solution (solution A) containing  $Na^+$ . Superfusion of cells with solution containing ATP (100  $\mu$ M) caused a rapid increase in the ratio (340/380 nm) of Fura-2 fluorescence ( $r_{\text{basal}} = 0.89 \pm 0.09$  to  $r_{\text{peak}} = 1.19 \pm 0.13$ ;  $n = 15$ ). However, the response was transient, and the  $[Ca^{2+}]_i$  returned close to basal value within 200 s after stimulation, even in the continuous presence of agonist ( $r = 0.92 \pm 0.09$ ;  $n = 15$ ) (Fig. 1A). Furthermore, when cells were exposed to ATP for the second time, only a small and even more transient change was detected in Fura-2 fluorescence (Fig. 1A). Administration of 10  $\mu$ M ATP caused a comparable but smaller change in  $[Ca^{2+}]_i$  (Table I). The effect of ATP was completely inhibited by the application of suramin (100  $\mu$ M) (Table I). ADP, 2MeSATP (100  $\mu$ M each), and ADP $\beta$ S (10 and 100  $\mu$ M) also caused an increase in cytosolic  $Ca^{2+}$  concentration, showing similar characteristics described for ATP (Fig. 1, B–D, and Table I). Because 2MeSATP and ADP $\beta$ S increased  $[Ca^{2+}]_i$  in a similar manner to ATP and ADP, these data argue strongly for activation of P2Y<sub>1</sub> receptors over other P2Y subtypes. In contrast, neither UTP (100  $\mu$ M) (Fig. 1A) nor UDP (100  $\mu$ M) had any effect on  $Ca^{2+}$  concentration (Table I). To explore whether degradation of ATP or ADP plays role in elevation of  $[Ca^{2+}]_i$ , we tested the effects of adenosine (100  $\mu$ M).

TABLE III  
Changes in Fura-2 fluorescence in IB3-1 cells 5 min after the peak stimulation

Δratios (340/380 nm) are changes in Fura-2 fluorescence 5 min after the peak stimulation *versus* unstimulated conditions. All values for % are percent changes in fluorescence *versus* ATP (100 μM) in Na<sup>+</sup>-containing medium. Values are means ± S.D.; n = number of experiments.

	Na <sup>+</sup> -containing medium			Na <sup>+</sup> -free medium		
	Δratio	%	n	Δratio	%	n
ATP (100 μM)	0.03 ± 0.02	100	15	0.10 ± 0.03 <sup>a</sup>	333	11
ATP (100 μM) + Ca <sup>2+</sup> -free media	No increase	0	5	No increase	0	3
ATP (100 μM) + ZnCl <sub>2</sub> (20 μM)	0.02 ± 0.01	66	4	0.26 ± 0.04 <sup>a,b</sup>	866	5
ATP (100 μM) at pH <sub>e</sub> = 7.9	0.04 ± 0.01	133	2	0.18 ± 0.02 <sup>a,b</sup>	600	5
ATP (100 μM) at pH <sub>e</sub> = 6.4	0.02 ± 0.01	66	2	0.02 ± 0.01	66	5
ATP (100 μM) + KB-R7943 (30 μM)	Not tested			0.14 ± 0.05 <sup>a</sup>	466	3
ATP (100 μM) + high [KCl] <sub>e</sub> (40 mM)	No increase	0	3	0.01 ± 0.01	33	4
ADPβS (100 μM)	0.01 ± 0.02	33	3	0.03 ± 0.01	100	3
BzBzATP (100 μM)	No increase	0	3	0.05 ± 0.01 <sup>a</sup>	167	5
ATP (100 μM) + 2APB (75 μM)	0.02 ± 0.02	66	2	0.12 ± 0.04 <sup>a</sup>	400	3
ATP (100 μM) + SKF-56365 (50 μM)	Not tested			0.25 ± 0.08 <sup>a,b</sup>	833	4

<sup>a</sup> p < 0.05 relative to ATP (100 μM) in Na<sup>+</sup>-containing medium.

<sup>b</sup> p < 0.05 relative to ATP (100 μM) in Na<sup>+</sup>-free medium.

Because adenosine did not increase [Ca<sup>2+</sup>]<sub>i</sub>, we did not pursue the participation of P1 receptors in increasing [Ca<sup>2+</sup>]<sub>i</sub> (Table I).

**Purinergic Agonists Trigger a Transient Increase in [Ca<sup>2+</sup>]<sub>i</sub> in the Absence of Extracellular Ca<sup>2+</sup>**—Activation of P2Y<sub>1</sub> receptors leads to G protein-coupled phospholipase C- and inositol 1,4,5-trisphosphate-dependent release of Ca<sup>2+</sup> from intracellular stores. As such, P2Y agonists should increase cytosolic Ca<sup>2+</sup> even in the absence of extracellular Ca<sup>2+</sup>. Therefore, we repeated the experiments with ATP (100 μM) (Fig. 2A) and ADP (100 μM) superfusing IB3-1 cells with solutions containing EGTA (1 mM) instead of CaCl<sub>2</sub> (solution B). Similar to control conditions, both agonists increased [Ca<sup>2+</sup>]<sub>i</sub> transiently, indicating that their effects, at least partially, were independent from extracellular Ca<sup>2+</sup> (Table I). Nonetheless, the absence of extracellular Ca<sup>2+</sup> reduced the agonist-induced peak increase in [Ca<sup>2+</sup>]<sub>i</sub> (Table I). Again, under these conditions, the Ca<sup>2+</sup> transients decayed fully back to base line within 200 s. Interestingly, these data did suggest that, besides P2Y<sub>1</sub> receptor activation, purinergic agonists may also trigger Ca<sup>2+</sup> influx from extracellular stores, which contributes to the peak increase in [Ca<sup>2+</sup>]<sub>i</sub>. Nevertheless, under these ionic conditions, Ca<sup>2+</sup> influx was not sufficient to support a sustained elevation of [Ca<sup>2+</sup>]<sub>i</sub>, the goal of this study. Experiments described below lend clarification to these early data.

**P2X Receptor-selective Agonists Fail to Trigger an Increase in [Ca<sup>2+</sup>]<sub>i</sub> in the Presence of Extracellular Na<sup>+</sup> and Ca<sup>2+</sup>**—Multiple subtypes of P2X receptors have already been described in human, rabbit, and rodent airway epithelial cells (27, 34, 35). Thus, we speculated that the higher peak in [Ca<sup>2+</sup>]<sub>i</sub> in the presence of extracellular Ca<sup>2+</sup> and the loss of the full response in Ca<sup>2+</sup>-free extracellular solution could be explained by the concomitant activation of P2X receptors activated by ATP. To test this hypothesis, we superfused IB3-1 cells with “solution A” containing either α,β-methylene ATP (α,β-MeATP, 100 μM) or benzoyl-benzoyl-ATP (BzBzATP, 100 μM) (Fig. 3A), selective agonists for different P2X receptor subtypes. Under these conditions, P2X-selective purinergic agonists failed to change [Ca<sup>2+</sup>]<sub>i</sub> (Table I). However, we were aware of the fact that α,β-MeATP and BzBzATP, although potent agonists at P2X<sub>1</sub>, P2X<sub>3</sub>, and P2X<sub>7</sub> receptors, have little or no effect at other P2XR subtypes. Thus, we hypothesized that changing the ionic composition of the superfusion medium might reveal activation of a Ca<sup>2+</sup> entry mechanism by these agonists (see below).

**ATP and BzBzATP Trigger an Increase in [Ca<sup>2+</sup>]<sub>i</sub> with Transient and Sustained Components in the Absence of Extracellular Na<sup>+</sup>**—Despite the negative data above with regard to P2X-selective agonists, we maintained the hypothesis that P2X receptors were involved in the full Ca<sup>2+</sup> response induced by

ATP in the presence of extracellular Ca<sup>2+</sup>. Rationale for this hypothesis is given by the fact that, in human and mouse lymphocytes, Na<sup>+</sup> might compete with Ca<sup>2+</sup> for entry through P2X receptors from extracellular stores (36–38) as well as other families of Ca<sup>2+</sup> entry channels like the transient receptor potential channels (TRPs) or the store-operated Ca<sup>2+</sup> channels (SOCs) (39, 40). Thus, we speculated that extracellular Na<sup>+</sup> might suppress the Ca<sup>2+</sup> permeability of P2X receptor channels in IB3-1 cells. To verify this hypothesis, we substituted extracellular Na<sup>+</sup> by N-methyl-D-glucamine (NMDG) (solution C) and tested the effects of a non-discriminant P2Y and P2X agonist (ATP), P2X-specific agonists (BzBzATP and α,β-MeATP), and a P2Y<sub>1</sub>-specific agonist (ADPβS). As shown in Fig. 2B (and in Fig. 5B and Fig. 7, A and B), substitution of extracellular Na<sup>+</sup> by NMDG itself caused a small but sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> ( $r_{\text{basal}} = 0.89 \pm 0.04$  to  $r_{\text{NMDG}} = 0.94 \pm 0.04$ ;  $n = 31$ ;  $p < 0.05$ ) which was completely absent when extracellular Ca<sup>2+</sup> was also omitted from the superfusion medium. These observations suggest the presence of a mechanism that allows sustained Ca<sup>2+</sup> entry, even in non-stimulated cells.

Following removal of extracellular Na<sup>+</sup> and changes in [Ca<sup>2+</sup>]<sub>i</sub>, we applied ATP (100 μM). Under these conditions, ATP induced a further increase in [Ca<sup>2+</sup>]<sub>i</sub> displaying a biphasic Ca<sup>2+</sup> response consisting of an initial transient peak and a sustained component (Fig. 2B and Tables II and III). In addition, as shown in Fig. 2B, a second application of ATP elicited a smaller increase in the [Ca<sup>2+</sup>]<sub>i</sub> peak; however, the sustained Ca<sup>2+</sup> plateau was comparable with that observed after the first stimulation by ATP. When [Ca<sup>2+</sup>]<sub>i</sub> reached a stable value after withdrawal of extracellular Na<sup>+</sup>, we also added either BzBzATP (100 μM) (Fig. 3B) or α,β-MeATP (100 μM). BzBzATP, but not α,β-MeATP, induced a small increase in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 3B and Tables II and III). This increase was completely dependent on the presence of extracellular Ca<sup>2+</sup>, indicating a role for P2X receptors in Ca<sup>2+</sup> influx (Fig. 3B and Table II). In Na<sup>+</sup>-free media, P2Y<sub>1</sub>-specific agonist, ADPβS (100 μM), augmented the peak increase in [Ca<sup>2+</sup>]<sub>i</sub> (Table II) but failed to elicit a sustained Ca<sup>2+</sup> plateau (Table III). Taken together, these data argue for a role for P2X receptors as Ca<sup>2+</sup> entry channels in IB3-1 cells.

**P2X<sub>4</sub> Receptor Channel Protein Biochemistry**—Due to the lack of other specific agonists or inhibitors, our functional studies did not distinguish further agonists among the P2XR subtypes. However, biochemical evidence suggests that IB3-1 cells express the P2X<sub>4</sub> receptor channel robustly. Membrane protein lysates from IB3-1 cells were prepared and were subjected to immunoblotting with a P2X<sub>4</sub>-specific polyclonal antibody. Fig. 4A shows the positive results for P2X<sub>4</sub> receptor channel protein

in total membrane protein lysates from IB3-1 cells grown on collagen-coated plastic as confluent monolayers. Inconsistent signals or a lack of signal was observed for P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>7</sub> using specific antibodies to those subtypes (data not shown). The P2X<sub>4</sub> signal displayed a similar biochemical phenotype compared with human vascular endothelial cells and human polycystic kidney disease renal epithelial cells performed in our laboratory (13, 41) as well as a recent study of P2X<sub>4</sub> receptor biochemistry in cardiac tissue and myocytes (42). An unglycosylated band was detected at ~46 kDa (the predicted molecular mass for P2X<sub>4</sub>) and a larger and broader glycosylated band at 60–65 kDa. These immunoblotting data show that P2X<sub>4</sub> is the most abundant P2X subtype expressed in IB3-1 cells. However, these data do not rule out less abundant expression of other P2X subtypes that is below the limit of detection with these antibodies. Further chemical modification of the extracellular solution also supports the abundant expression of P2X<sub>4</sub> receptor channels as the major P2X receptor subtype mediating Ca<sup>2+</sup> entry (see below).

Fig. 4, B–D, shows additional data in 16HBE140<sup>−</sup> non-CF airway epithelial cells. Immunoblotting of non-polarized cells grown in flasks (Fig. 4, B and C) as well as biotinylation (Fig. 4D) of polarized monolayers grown on permeable supports revealed robust and apical membrane-localized expression of P2X<sub>4</sub>. In these lysates, a third band of ~100 kDa was also found. Biotinylation was performed on the apical and basolateral surface of these monolayers. Only the apical signal is shown in Fig. 4D, although a detectable signal was also observed in basolateral biotinylated material (data not shown). Secondary antibody controls and blocking of antibody binding with the peptide immunogen, provided with the primary antibody in all biochemical assays, verified the specificity of P2X<sub>4</sub> receptor expression (data not shown). These data suggest that P2X<sub>4</sub> receptors are expressed abundantly by human airway epithelial cells grown under non-polarized and polarized conditions.

**The Extracellular ATP-gated P2X<sub>4</sub> Receptor Channel Is the Major Ca<sup>2+</sup> Entry Channel Stimulated by ATP in IB3-1 and 16HBE140<sup>−</sup> Cells**—Like other subtypes of the P2X receptor channel family, the P2X<sub>4</sub> receptors are also regulated by different cations, such as H<sup>+</sup> or Zn<sup>2+</sup> (25). Thus, if it is true that in IB3-1 cells the prolonged Ca<sup>2+</sup> response in Na<sup>+</sup>-free medium was due to activation of P2X<sub>4</sub> receptors, then extracellular pH and Zn<sup>2+</sup> should modify the ATP-induced Ca<sup>2+</sup> signal. To test this hypothesis, we measured [Ca<sup>2+</sup>]<sub>i</sub> after changing extracellular pH or in the presence of Zn<sup>2+</sup> in both IB3-1 and 16HBE140<sup>−</sup> cells. We exposed IB3-1 cells to ATP after changing the pH of the superfusion solution. As shown in Table III, increasing extracellular pH potentiated the ATP-induced sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> only in Na<sup>+</sup>-free medium. Furthermore, in a Na<sup>+</sup>-free environment, acidic pH significantly reduced the ATP-induced peak increase in [Ca<sup>2+</sup>]<sub>i</sub> (Table II). To demonstrate directly the effect of ATP on Ca<sup>2+</sup> influx from extracellular sources via another approach, we measured quenching of Fura-2 at 359 nm in the presence of MnCl<sub>2</sub> (500 μM). Mn<sup>2+</sup> is known to permeate the same entry channels as Ca<sup>2+</sup> and quenches Fura-2 fluorescence when it enters the cells. As shown in Fig. 5A, in Na<sup>+</sup>-free medium, acidic extracellular pH (6.4) inhibited Mn<sup>2+</sup> entry, whereas alkaline extracellular pH (7.9) potentiated markedly Mn<sup>2+</sup> entry and quenching of the dye. To further support the involvement of P2X<sub>4</sub> receptor channels, we tested the effect of the P2X receptor co-agonist, Zn<sup>2+</sup>, on ATP-induced Ca<sup>2+</sup> entry mechanisms. Inclusion of ZnCl<sub>2</sub> (20 μM) further augmented the sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> induced by ATP in Na<sup>+</sup>-free medium (Fig. 5B and Table III) but had no effect in Na<sup>+</sup>-containing medium

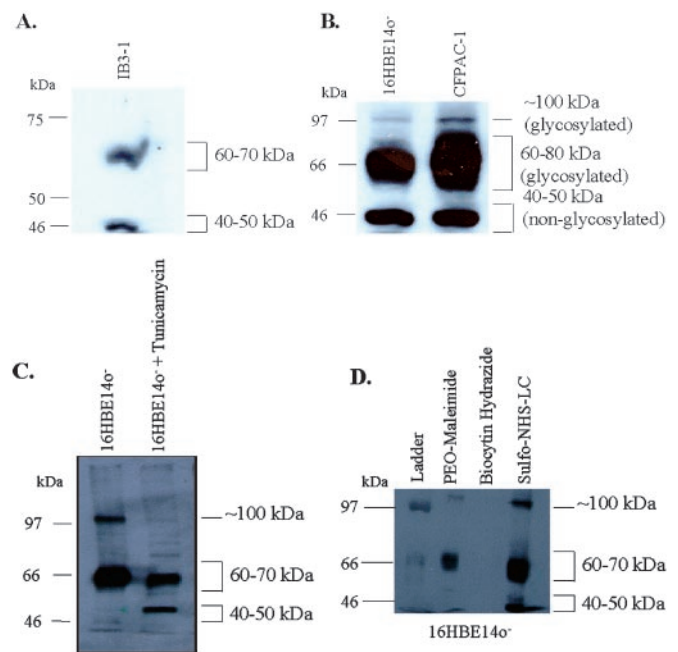


FIG. 4. A, immunoblot analysis of IB3-1 cells grown as non-polarized monolayers in flasks using rabbit polyclonal antibodies against P2X<sub>4</sub> receptors. A smaller band of the predicted molecular mass for P2X<sub>4</sub> (46 kDa) was detected, as was a larger, broader, glycosylated band at 60–70 kDa. The positions of molecular mass markers are shown on the left (in kDa). This is representative of 3 such experiments. B, immunoblot analysis of 16HBE140<sup>−</sup> cells and CFPAC-1 cells grown as polarized cell monolayers (CFPAC-1 cells were screened as another CF-relevant cell line and were grown in a similar manner than 16HBE140<sup>−</sup> cells except that Iscove's modified essential medium was used for the basal medium with all other additives kept similar). Note the stronger expression in polarized cell monolayers and the presence of a 40–50-kDa band (unglycosylated predicted molecular mass), a 60–80-kDa band (glycosylated form), and an even larger form at ~100 kDa (glycosylated form). This is representative of 6 such experiments. C, tunicamycin (10 μM), an inhibitor of glycosylation, added to the culture medium in an overnight 24-h incubation of confluent cell monolayers grown in flasks abolished the 100-kDa form and inhibited the expression of the 60–80-kDa band, yielding more of the 40–50-kDa unglycosylated form. This is representative of 2 such experiments. D, three water-soluble forms of biotin reagents were used to biotinylate apical membrane P2X<sub>4</sub>. The data reveal that poly(ethylene)oxid-maleimide biotin, a reagent that reacts with primary amines primarily on lysine residues, detected only the glycosylated forms in the apical plasma membrane of 16HBE140<sup>−</sup> epithelial cell monolayers. Biocytin hydrazide failed to work in this experiment, likely because our conditions for oxidizing the carbohydrate residues were not optimal. Sulfo-NHS-LC-biotin detected all of the forms, indicating that it may have detected apical P2X<sub>4</sub>; however, it may have gained access to the cell interior to find the unglycosylated form as well. This is representative of 2 such experiments. Note pertaining to all panels: no secondary antibody controls were performed for all of the above experiments, as were peptide immunogen blocking experiments that effectively blocked the signal. Peptide immunogens for P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>7</sub> did not block the P2X<sub>4</sub> signaling, revealing additional specificity. In addition to data from our laboratory in human ADPKD kidney epithelial cells (41) and human vascular endothelial cells (13), this is the first documentation of biochemical detection of native airway epithelial P2X<sub>4</sub> receptor protein.

(Table III). Since our biochemical data (see above) indicated that P2X<sub>4</sub> receptors are also present in 16HBE140<sup>−</sup> non-CF airway epithelial cells, we tested whether increasing extracellular pH or addition of Zn<sup>2+</sup> augmented the ATP-induced sustained Ca<sup>2+</sup> entry in Na<sup>+</sup>-free medium in 16HBE140<sup>−</sup> cells. As shown in Fig. 6A, ATP elicited extracellular pH-dependent quenching of Fura-2, suggesting that ATP-stimulated Ca<sup>2+</sup> influx is facilitated by alkaline pH. In addition, similar to results obtained with IB3-1 cells, both inclusion of Zn<sup>2+</sup> and increasing pH potentiated the effects of ATP on sustained Ca<sup>2+</sup> signal (Fig. 6B). Taken together, these data argue for a prom-



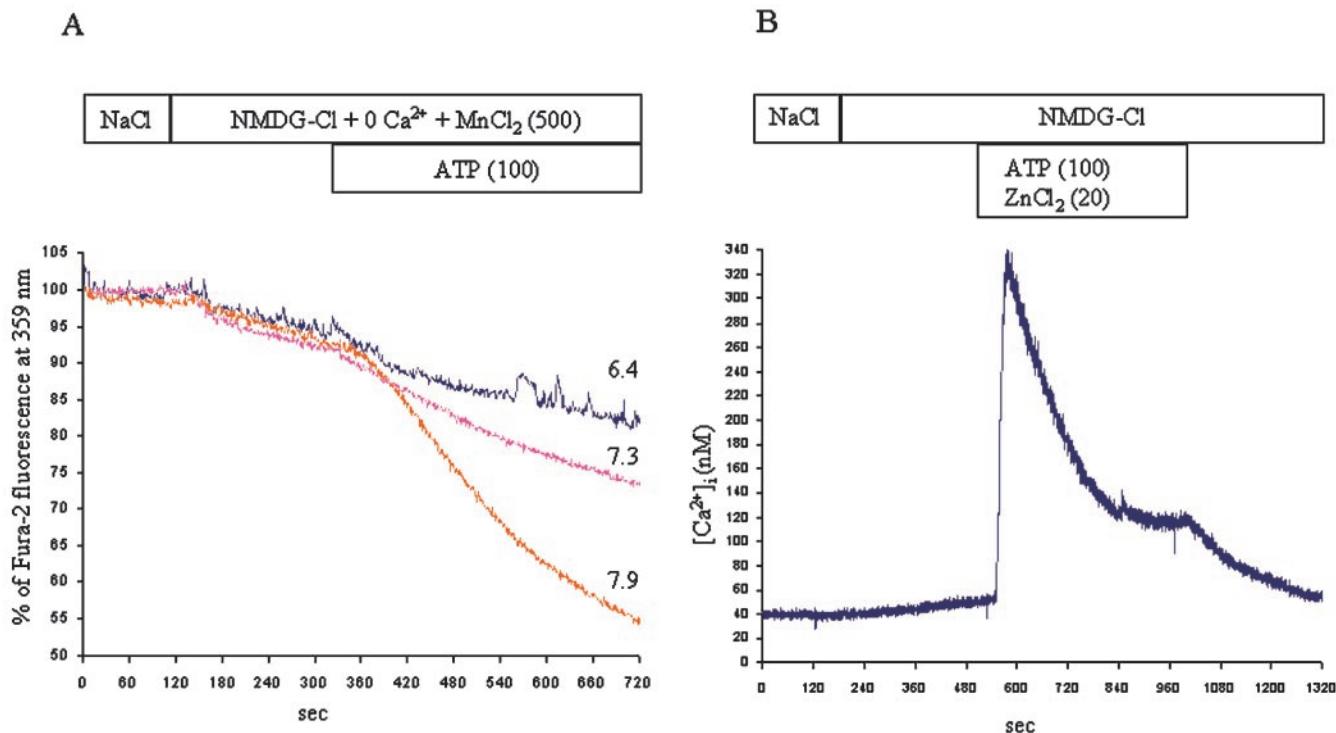


FIG. 5. Representative traces showing the pH dependence of ATP-induced  $Mn^{2+}$  entry in IB3-1 cells. A, quenching of Fura-2 was measured at the isosbestic wavelength of Fura-2 (359 nm). Cells were exposed to  $MnCl_2$  (500  $\mu M$ ) in  $Na^+$ - and  $Ca^{2+}$ -free medium at pH 7.3. After 200 s, ATP (100  $\mu M$ ) was added to the superfusion medium having three different pH values, as indicated. At least 3 experiments have been done in each group with similar results. A representative trace shows the effects of ATP (100  $\mu M$ ) in presence of  $ZnCl_2$  (20  $\mu M$ ) in cells exposed to  $Na^+$ -free medium (B) as indicated. Please note the augmentation of the sustained plateau of increased  $[Ca^{2+}]_i$  in IB3-1 cells by inclusion of  $ZnCl_2$  (compare with original trace in Fig. 2B).

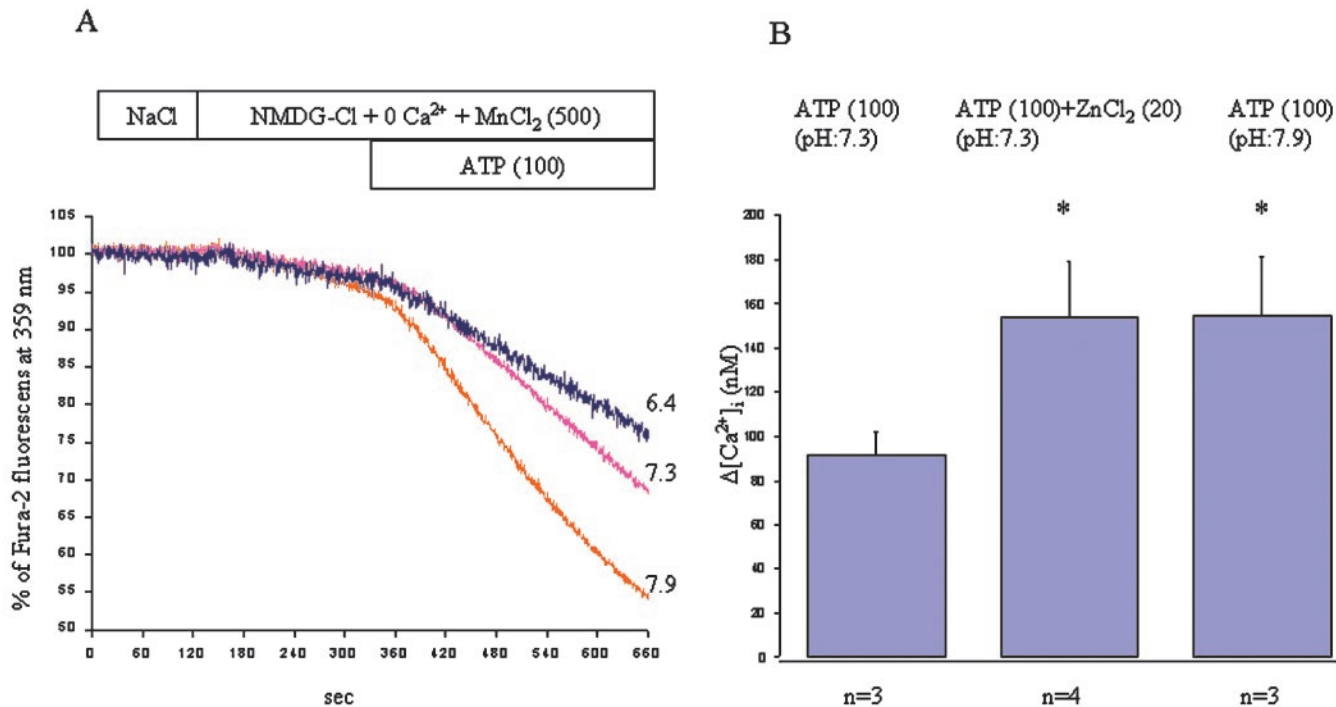


FIG. 6. Representative traces on the left showing the pH dependence of ATP-induced  $Mn^{2+}$  entry in 16HBE14o $^-$  cells. A, experiments were performed in a similar manner to those in Fig. 5A. Changes in cytosolic  $Ca^{2+}$  concentration 5 min after the peak stimulation versus the basal  $[Ca^{2+}]_i$  in 16HBE14o $^-$  cells are shown in B. Effects on the sustained plateau of increased  $[Ca^{2+}]_i$  in 16HBE14o $^-$  cells are shown illustrating the potentiating effect of  $ZnCl_2$  and of alkaline pH. All experiments have been done in  $Na^+$ -free medium. \*,  $p < 0.05$

inent role for the  $P2X_4$  receptor as a  $Ca^{2+}$  entry channel in human airway epithelial cells and argue against a functional role for other  $P2X$  receptor subtypes.  
*The  $P2X_4$ -mediated  $Ca^{2+}$  Entry Is Sustained, Long Lived,*

*Reversible, and Re-acquired upon Re-addition of Agonist*—For any therapeutic approach to be effective, especially one that targets an endogenous receptor, stimulation should be sustained and long lived. Even more desirable, the effect should be

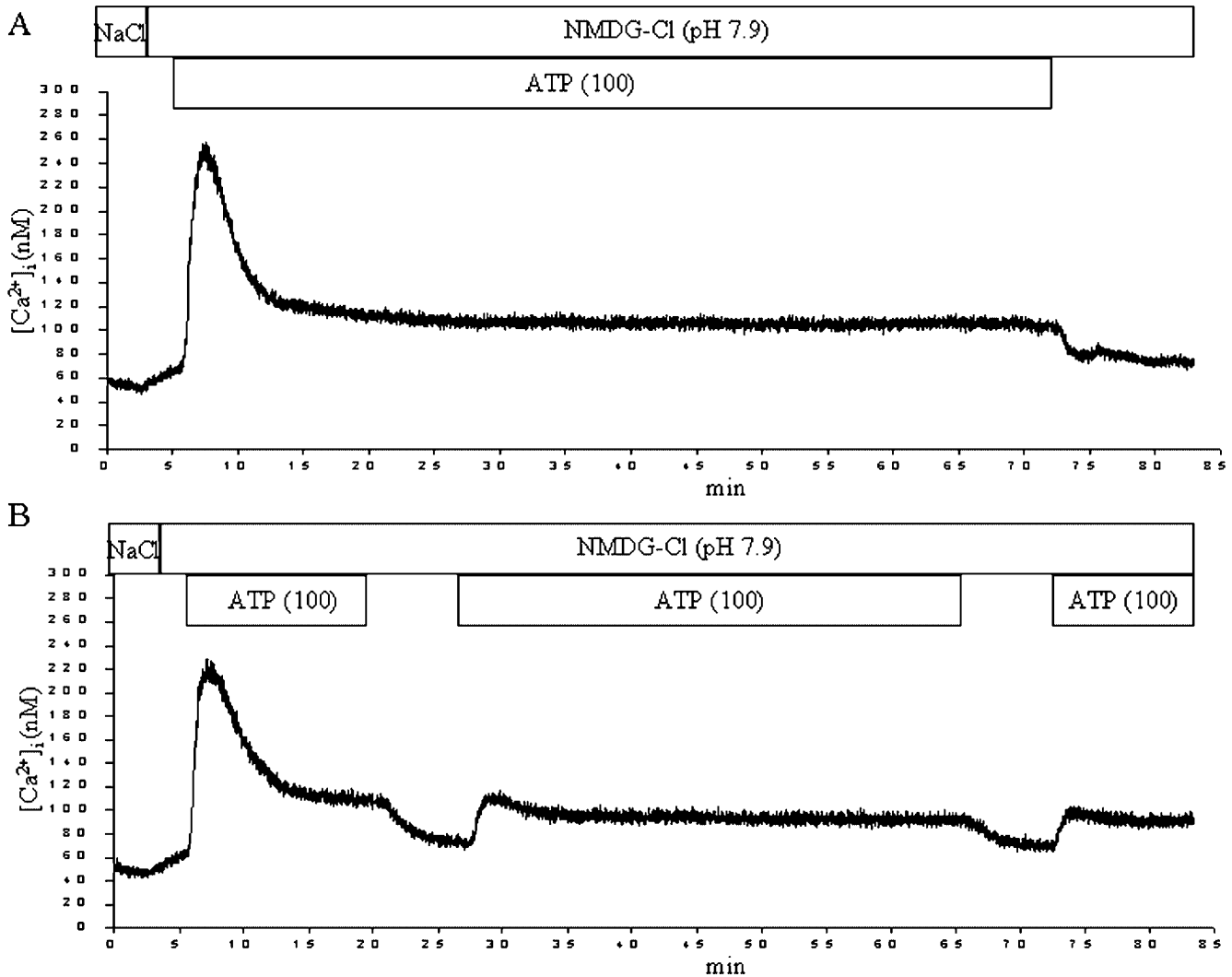


FIG. 7. Representative traces showing the duration of the sustained plateau in  $[Ca^{2+}]_i$  in IB3-1 CF cells induced by ATP under  $Na^+$ -free conditions (pH 7.9) (A) and the reversibility, long lived, and reproducible nature of the sustained plateau induced by ATP and mediated by  $P2X_4$  (B). Each trace for each protocol is typical of 3 such experiments.

reversible to control the response. Ultimately, it is ideal if this endogenous receptor target did not desensitize or inactivate, as is apparent in this study for  $P2Y$ -mediated transient  $Ca^{2+}$  signal. Fig. 7 shows experiments designed to determine whether  $P2X_4$ -mediated  $Ca^{2+}$  entry was sustained and long lived in IB3-1 cells. In the first protocol, ATP (100  $\mu$ M) was added in  $Na^+$ -free solution that has pH 7.9. A transient increase in  $[Ca^{2+}]_i$  mediated by  $P2Y$  receptors was followed by a sustained plateau that persisted for over 60 min, until ATP was removed (Fig. 7A). In a second approach, a 15-min stimulation was performed with ATP and then was reversed with washout. Following re-addition of ATP, a similar sustained calcium plateau was acquired that persisted for 40 min. A third washout and stimulation was performed at the end of the protocol (Fig. 7B), showing lack of desensitization of the  $P2X_4$  receptors or inactivation of their channel function. In contrast, the transient spike observed in the first application of ATP was lost. These data show, these data show that the  $P2X_4$ -mediated  $Ca^{2+}$  entry is sustained, long lived, reversible, and re-acquirable upon washout and re-addition of agonist.

*Neither the Reverse Operation Mode of the  $Na^+/Ca^{2+}$  Exchanger Nor Voltage-dependent  $Ca^{2+}$  Channels or Store-operated  $Ca^{2+}$  Channels Are Involved in ATP-induced  $Ca^{2+}$  Entry in IB3-1 Cells*—Theoretically, both the initial increase in

$[Ca^{2+}]_i$  after removal of extracellular  $Na^+$  and the sustained  $Ca^{2+}$  plateau induced by administration of ATP could be due to the activation of the  $Na^+/Ca^{2+}$  exchanger in its reverse operation mode and/or other classes of  $Ca^{2+}$  entry channels. Thus, we removed extracellular  $Na^+$  and added ATP in the presence of KB-R7943 (30  $\mu$ M), a specific inhibitor of reverse operation mode of the  $Na^+/Ca^{2+}$  exchanger (43). Since KB-R7943 had no effect under these experimental conditions, we excluded the presence of this exchanger at the plasma membrane (Fig. 8A and Tables II and III). Although airway epithelial cells are non-excitable cells and should not express voltage-dependent  $Ca^{2+}$  channels, we asked the question whether cell membrane depolarization stimulated or inhibited the  $Ca^{2+}$  response induced by ATP. Therefore, we exposed the cells to high extracellular KCl concentration (40 mM) in  $Na^+$ -free medium (solution D), and then we added ATP. As shown in Fig. 8B and Tables II and III, membrane depolarization inhibited the peak increase of  $[Ca^{2+}]_i$ , and the sustained  $Ca^{2+}$  plateau was completely abolished, indicating that IB3-1 cells do not express voltage-dependent  $Ca^{2+}$  channels.

SOCs or TRPs represent other pathways by which  $Ca^{2+}$  can enter non-excitable cells besides the ATP-gated  $P2X$  receptor channels. Theoretically, both SOC and TRPs could be responsible for the sustained  $Ca^{2+}$  influx induced by ATP in  $Na^+$ -free



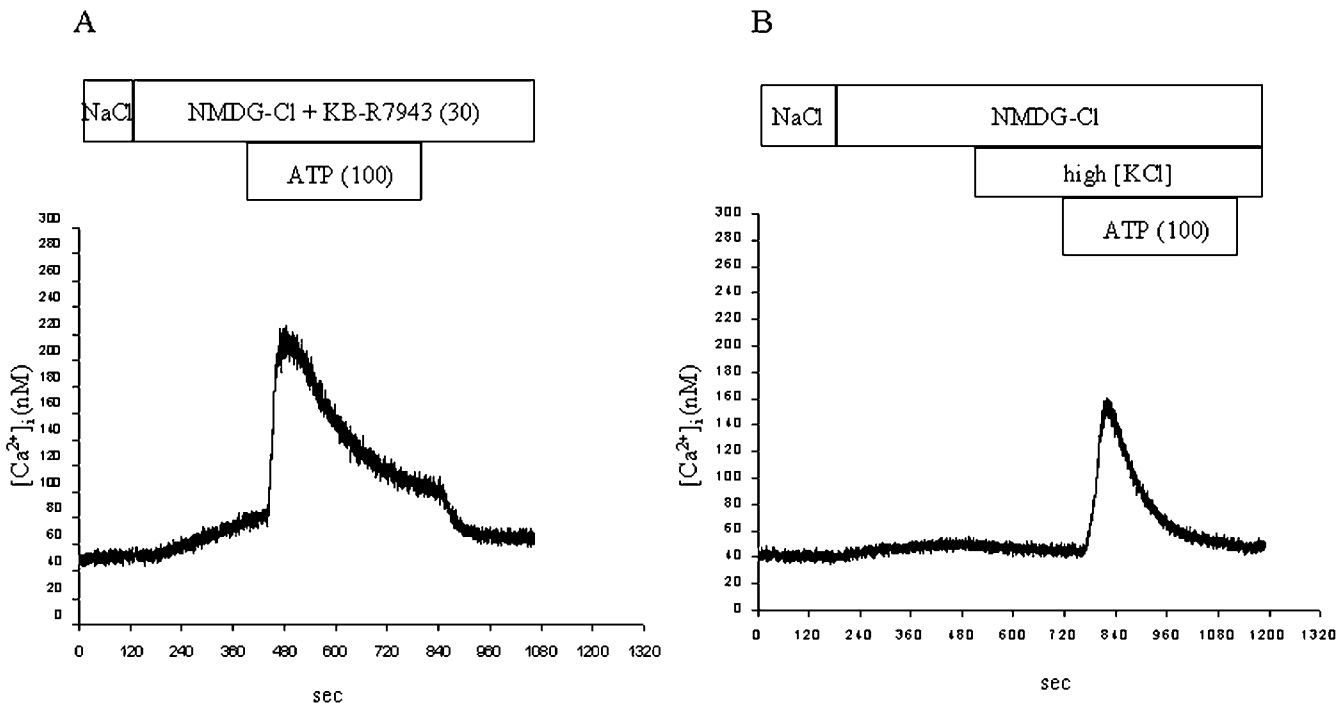


FIG. 8. Representative traces showing the effects of KB-R7943 (30  $\mu$ M) (A) and high  $[KCl]_e$  (40 mM) (B) on ATP-induced  $Ca^{2+}$  signal. Experiments were done in a  $Na^+$ -free environment. B, note that substitution of  $Na^+$  by NMDG causes a slight increase in  $[Ca^{2+}]_i$ , an effect that was inhibited in high KCl-containing solution.

medium. Therefore, we tested whether SOC are present in IB3-1 cells. We treated the cells with thapsigargin (100 nM), an inhibitor of  $Ca^{2+}$  pump in the ER membrane, in the presence of extracellular  $Ca^{2+}$ . This maneuver induced a large initial increase in Fura-2 fluorescence ratio ( $r_{\text{basal}} = 1.00 \pm 0.05$  to  $r_{\text{peak}} = 2.92 \pm 0.17$ ;  $n = 3$ ) followed by a sustained  $Ca^{2+}$  plateau ( $r_{\text{sustained}} = 1.58 \pm 0.29$ ;  $n = 3$ ). In the absence of extracellular  $Ca^{2+}$ , stimulation with thapsigargin resulted in a small transient increase in  $[Ca^{2+}]_i$  due to the depletion of intracellular  $Ca^{2+}$  stores, and the re-addition of extracellular  $Ca^{2+}$  elicited a large  $[Ca^{2+}]_i$  increase (Fig. 9). These data indicate that IB3-1 cells possess SOC, which are activated by a decrease in  $[Ca^{2+}]_{ER}$ . Next, we have asked whether SOC or store-independent TRP-like channels contribute to the sustained  $Ca^{2+}$  increase after  $P2Y_1$  receptor stimulation in  $Na^+$ -free medium. To address this question, we used 2APB, which has recently been reported to inhibit SOC (44, 45), and SKF-96365, which is a blocker of the store-independent TRPs (46). Neither 2APB (75  $\mu$ M) nor SKF-56365 (50  $\mu$ M) abolished the ATP-induced sustained increase in  $[Ca^{2+}]_i$  in the absence of extracellular  $Na^+$  (Table III). Interestingly, the sustained  $Ca^{2+}$  plateau was further augmented by the SKF-96365 compound (Table III). These data indicate that, in IB3-1 cells, SOC and/or TRPs do not play a role in regulating  $[Ca^{2+}]_i$  following purinergic receptor stimulation.

DISCUSSION

Stimulation of purinergic receptors exerts biological effects, which are mediated in part through elevation of intracellular  $Ca^{2+}$  concentration (47–52). In the present study, we show evidence that IB3-1 cells express  $P2Y_1$  and  $P2X_4$  receptors abundantly.  $P2Y_1$  receptors have been found recently in airway epithelia of  $P2Y_2$  receptor-knockout mice (54), in rat lung (55), and in Calu-3 human airway epithelial cells (56). ADP $\beta$ S, a specific agonist of  $P2Y_1$  receptors, increased  $[Ca^{2+}]_i$  to a similar extent as ATP, ADP, and 2MeSATP, suggesting the presence of  $P2Y_1$  receptors. Although recent data (57) indicate that 2MeSATP and, possibly, ADP $\beta$ S at a concentration of 100  $\mu$ M may

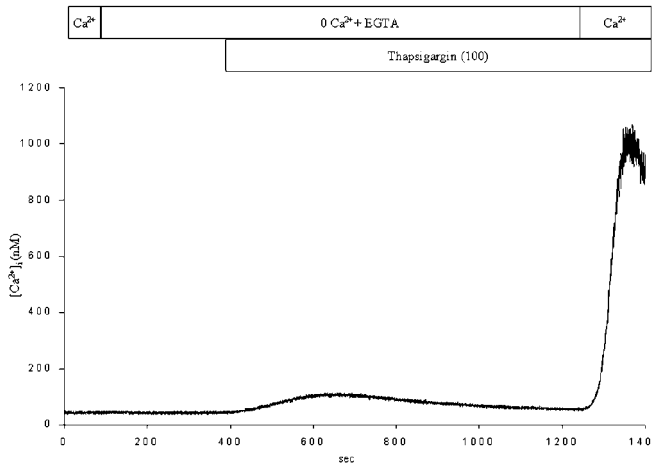


FIG. 9. Representative trace shows the effect of thapsigargin (100 nM) on  $[Ca^{2+}]_i$  in the absence and in the presence of extracellular  $Ca^{2+}$  as indicated. This maneuver reveals the presence of SOC involved in  $Ca^{2+}$  entry, albeit induced by emptying ER stores completely.

activate  $P2Y_{11}$  receptors, we believe it is very unlikely that the increase in  $[Ca^{2+}]_i$  observed in this study was due to the activation of  $P2Y_{11}$  receptors. This conclusion derives from the fact that  $P2Y_{11}$  receptors are poorly stimulated by ADP (26), whereas our data show that ADP is at least as potent an agonist as ATP. In addition, ADP $\beta$ S also elicited a significant increase in  $[Ca^{2+}]_i$  at a concentration of 10  $\mu$ M. In other airway epithelial cell models, the presence of  $P2Y_2$  has already been demonstrated (16, 58, 59). Furthermore, *in vivo* studies demonstrate that aerosolized UTP has beneficial effects in treatment of CF lung disease, confirming the presence of  $P2Y_2$  and/or  $P2Y_4$  on the apical membrane of airway epithelium (23, 48). Interestingly, neither UTP nor UDP increased  $[Ca^{2+}]_i$  in IB3-1 cells; however, both agonists do rescue impaired cell

volume regulation in IB3-1 cells.<sup>2</sup> These differences may reveal additional signal transduction pathways triggered by P2Y receptors that are independent of cytosolic calcium.

Nevertheless, in addition to the beneficial targeting of P2Y receptors for CF therapy, we argue here for the beneficial targeting of P2X receptors as well. Activation of these receptors would also have the added benefit of eliciting a sustained increase in  $[Ca^{2+}]_i$ , an effect not observed with P2Y-specific agonists. The transient nature of the  $Ca^{2+}$  signal induced by purinergic agonists accounts presumably for transient  $Cl^-$  and fluid secretion observed in different CF epithelial cell models (7, 29). Activation of P2X receptor channels under appropriate conditions would lead to  $Ca^{2+}$  influx from the extracellular space. Furthermore, this  $Ca^{2+}$  response is sustained for at least 1 h, is reversible, and is re-acquired to the same sustained level upon re-addition of agonists under conditions designed to stimulate P2X<sub>4</sub>.

However, our data could conceivably be explained in the following ways: 1) opening of extracellular ATP-gated P2X receptor channels; 2) activation of  $Na^+/Ca^{2+}$  exchanger in reverse operation mode due to  $Na^+$  removal; 3) opening of voltage-dependent  $Ca^{2+}$  channels following membrane depolarization; and 4) activation of SOCs or TRPs after depletion of intracellular  $Ca^{2+}$  stores. All lines of evidence indicate that activation of ATP-gated P2X<sub>4</sub> receptor channels led to augmentation of  $Ca^{2+}$  signal and the sustained  $Ca^{2+}$  plateau. First, in IB3-1 cells, BzBzATP, a P2X receptor-specific agonist, increases  $[Ca^{2+}]_i$  only in  $Na^+$ -free medium. Second, the ATP-induced  $Ca^{2+}$  plateau was enhanced by alkaline extracellular pH and inhibited by acidic extracellular pH. Third, ATP-induced  $Mn^{2+}$  entry caused quenching of Fura-2 in a pH-dependent manner exhibiting significant increase in  $Mn^{2+}$  permeability at alkaline pH. Fourth, application of  $Zn^{2+}$  further enhanced the effects of ATP. Fifth, a P2Y<sub>1</sub> receptor-specific agonist, ADP $\beta$ S, did not cause a sustained increase in  $[Ca^{2+}]_i$ . Sixth, neither 2APB, an inhibitor of SOCs, nor SKF-56365, a blocker of store-independent TRP-like channels, abolished the sustained increase in  $[Ca^{2+}]_i$  induced by ATP. Seventh, recent data (60, 61) indicate that  $Zn^{2+}$  inhibits SOCs. Eighth, biochemical evidence showed abundant expression of P2X<sub>4</sub>. Roles for the reverse mode of the  $Na^+/Ca^{2+}$  exchanger and/or voltage-dependent  $Ca^{2+}$  channels were ruled out with a variety of different cell biological maneuvers and/or pharmacological inhibitors. It is noteworthy that Vennekens *et al.* (62) have recently reported that epithelial  $Ca^{2+}$  channels are regulated by extracellular pH. However, these channels are mainly expressed in kidney and intestinal epithelia and inhibited by metal ions at low micromolar concentration (63).

Although BzBzATP is primarily known to be an agonist of P2X<sub>7</sub> and antibodies used in this study were raised against rat P2X receptors, stimulation by  $Zn^{2+}$  and inhibition by  $H^+$  are most consistent with activation of the P2X<sub>4</sub> receptors and inconsistent with other P2X receptor subtypes (25). For instance, stimulatory effects by  $Zn^{2+}$  rule out a role for P2X<sub>7</sub>, because  $Zn^{2+}$  is a P2X<sub>7</sub> antagonist (25). Inhibition of  $Ca^{2+}$  entry by acidic pH rules out P2X<sub>2</sub> receptors, which are stimulated by acidic pH (25). The only phenotype that is not completely explained by P2X<sub>4</sub> alone is the alkaline pH stimulation. Heterologously expressed P2X<sub>4</sub> is only mildly stimulated by alkaline pH (64). As such, we cannot rule out that additional P2X receptor subtypes (perhaps P2X<sub>5</sub> (65), P2X<sub>6</sub> (66), or splice variants of P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>6</sub> (67)) may be conferring these pH effects in a P2XR heteromultimer. Interestingly, in 16HBE140<sup>-</sup> cells, ATP-driven  $Mn^{2+}$  entry was also enhanced by alkaline pH, and  $Zn^{2+}$  potentiated the ATP-induced sus-

tained increase in  $[Ca^{2+}]_i$ . Taken together, these data indicate that P2X<sub>4</sub> receptors function as ATP-gated  $Ca^{2+}$  entry channels in both CF and non-CF airway epithelial cells.

In a past study (27), our laboratory showed that a P2X-selective agonist, BzBzATP, stimulated transepithelial chloride secretion in Ussing chamber experiments on airway epithelia that had both transient and sustained components and in nasal potential difference assays on mouse nasal mucosa that were transient stimulations that averaged 1–2 mV. These stimulations occurred in  $Na^+$ -rich solutions (27). Despite this knowledge, we did not perform experiments designed to examine P2XR-mediated signaling in this study (27). Because  $Na^+$  is in great excess to  $Ca^{2+}$  in physiological saline, the contribution of  $Ca^{2+}$ -permeable non-selective cation channels to a  $Ca^{2+}$  entry phenotype is often masked. This was true for our CF cell model. In IB3-1 cells, removal of extracellular  $Na^+$  was required to observe any increase in  $[Ca^{2+}]_i$  with BzBzATP and a sustained  $Ca^{2+}$  signal with ATP. Nonetheless, in 16HBE140<sup>-</sup> non-CF cells, extracellular  $Na^+$  (140 mM) prevented neither the BzBzATP-dependent  $Ca^{2+}$  response nor the ATP-induced  $Ca^{2+}$  plateau<sup>3</sup>; however, responses to both BzBzATP and ATP were much more profound under  $Na^+$ -free conditions. Thus, we speculate that P2XR agonists might be useful in CF therapy regardless of extracellular  $Na^+$  concentration, although modification of the extracellular environment ( $Na^+$  removal, among other maneuvers) may strengthen their efficacy and was required to optimally study  $Ca^{2+}$  entry mechanisms in Fura-2 spectrofluorometry. Nevertheless, further studies are required to determine whether the presence of extracellular  $Na^+$  inhibits P2XR-mediated rescue of  $Cl^-$  secretion in CF therapy.

Interestingly, although controversial, recent data indicate that airway surface liquid (ASL) in non-CF subjects is hypotonic and low in  $Na^+$  with respect to the plasma (68). In contrast, other studies (69) have concluded that non-CF and CF ASL are isotonic. Nevertheless, it is noteworthy that, in  $Na^+$ -replete medium, extracellular ATP stimulation of ciliary beat is attenuated, whereas in  $Na^+$ -free medium, ATP induction of ciliary beat was profound, suggesting a role for P2X receptors on cilia (35). Because cilia reside and need to function optimally in the ASL environment, we postulate that normal ASL may be hypotonic and, in particular, low in  $Na^+$ , allowing P2X receptor agonists to stimulate sustained signaling that may impact ion transport and ciliary beat. These specialized chemical and ionic conditions may also be critical in the delivery of agonists for CF therapy. This is tenable, because the vehicle for delivery during nebulization, aerosolization, or instillation would merely need to be modified to suit these optimal conditions.

Taken together, these findings are profound with regard to therapy in CF, because they suggest that endogenously expressed P2X receptors do not desensitize or inactivate, and under appropriate conditions, their activation leads to a prolonged  $Ca^{2+}$  signal that could translate into a sustained  $Cl^-$  secretion in CF and non-CF epithelia.

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<sup>2</sup> G. M. Braunstein and E. M. Schwiebert, unpublished observations.

<sup>3</sup> A. Zsembery and E. M. Schwiebert, unpublished observations.

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# Bicarbonate Inhibits Bacterial Growth and Biofilm Formation of Prevalent Cystic Fibrosis Pathogens

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We investigated the effects of bicarbonate on the growth of several different bacteria as well as its effects on biofilm formation and intracellular cAMP concentration in *Pseudomonas aeruginosa*. Biofilm formation was examined in 96-well plates, with or without bicarbonate. The cAMP production of bacteria was measured by a commercial assay kit. We found that NaHCO<sub>3</sub> (100 mmol l<sup>-1</sup>) significantly inhibited, whereas NaCl (100 mmol l<sup>-1</sup>) did not influence the growth of planktonic bacteria. MIC and MBC measurements indicated that the effect of HCO<sub>3</sub><sup>-</sup> is bacteriostatic rather than bactericidal. Moreover, NaHCO<sub>3</sub> prevented biofilm formation as a function of concentration. Bicarbonate and alkalinization of external pH induced a significant increase in intracellular cAMP levels. In conclusion, HCO<sub>3</sub><sup>-</sup> impedes the planktonic growth of different bacteria and impedes biofilm formation by *P. aeruginosa* that is associated with increased intracellular cAMP production. These findings suggest that aerosol inhalation therapy with HCO<sub>3</sub><sup>-</sup> solutions may help improve respiratory hygiene in patients with cystic fibrosis and possibly other chronically infected lung diseases.

**Keywords:** cystic fibrosis, bicarbonate, pH, biofilm, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, cAMP, HCO<sub>3</sub><sup>-</sup>

## INTRODUCTION

Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Riordan et al., 1989). CFTR is a cAMP/protein kinase A (PKA)-dependent epithelial anion channel that conducts both chloride and bicarbonate (Linsdell et al., 1997; Reddy and Quinton, 2003). Defective transepithelial anion transport impairs mucociliary clearance (MCC) leading to the retention of thick, viscid mucus in the airways (Quinton, 2007a, 2010). The poor clearance of viscous CF mucus contributes to a vicious cycle of airway obstruction, infection, and inflammation (Hoffman and Ramsey, 2013). However, the links between the primary defect in anion transport and CF lung disease appear to be multifactorial. It was recently shown that impaired HCO<sub>3</sub><sup>-</sup> secretion is likely responsible for aggregated mucus in CF mice (Garcia et al., 2009; Gustafsson et al., 2012) and pigs (Birket et al., 2014).

In addition, the abnormally lower pH of the airway surface liquid (ASL) was associated with decreased bacterial killing in the CF porcine lung. Aerosolizing a HCO<sub>3</sub><sup>-</sup> solution onto the CF porcine airways increased innate bacterial killing *in vivo* (Pezzulo et al., 2012). Recruitment of

lymphocytes may promote epithelial  $\text{HCO}_3^-$  secretion during infection and, intriguingly, epithelial  $\text{HCO}_3^-$  secretion may require CFTR expression in lymphocytes as yet another deficiency impacting on CF lung disease (Tang et al., 2012).

For decades,  $\text{HCO}_3^-$  has been used and indicated for use as a microbial disinfectant in food and agriculture industries, but usually as an adjuvant (Rutala et al., 2000). Earlier,  $\text{HCO}_3^-$ , in combination with lidocaine was reported to exert antibacterial activity (Thompson et al., 1993). Immunologically, it is crucial for the optimal activity of antimicrobial peptides (Dorschner et al., 2006) but, apparently, it can have an independent bactericidal effect on *Escherichia coli* (Xie et al., 2010). In medicine, it is an accepted treatment for chlorine gas intoxication (Bosse, 1994) and has been suggested for use as a mucolytic as well as an adjuvant for nebulized drug delivery (Kaushik et al., 2016). It has also been long recommended for dental hygiene (Newbrun et al., 1984; Drake et al., 1995). Most of the antibacterial reports on  $\text{HCO}_3^-$  have been limited to planktonic growth and do not include effects on biofilm formation.

Bicarbonate directly and indirectly affects lung function (Quinton, 2007a,b). It is required for sequestering calcium and protons from secreted mucin granules to allow for normal expansion upon release (Garcia et al., 2009; Quinton, 2010). It may also affect neutrophil killing capacity as well as bacterial colonization in the lungs (Quinton, 2007a, 2008; Abou Alaiwa et al., 2014). Importantly,  $\text{HCO}_3^-$  influences the  $\text{H}^+$  concentration (pH) of the ASL via the  $\text{HCO}_3^-/\text{CO}_2$  buffer system (Shah et al., 2016a). These effects directly affect the properties of the ASL and its critical ability to trap inhaled and endogenous debris for export by the ciliated surfaces of the airways. They indirectly affect the lungs by maintaining an airway environment that also enables the immune system to clear viral and bacterial pathogens. Any maneuver such as adding exogenous  $\text{HCO}_3^-$ , as suggested here, that improves or helps maintain airway patency is expected to enhance lung function.

Successful antimicrobial therapy for bacterial lung infections is crucial for increasing life expectancy and improving CF patients' quality of life. In CF lungs, however, bacteria colonize in biofilms (Rogers et al., 2011), making eradication of pathogens difficult. It is also known that bacteria in biofilms are more resistant compared to planktonic cells (Stewart and Costerton, 2001; Venkatesan et al., 2015). Biofilm formation is thought to be regulated largely by the second messenger molecules cAMP and c-di-GMP in bacterial cells. An increase in intracellular cAMP concentrations along with a decrease in c-di-GMP levels is associated with the production of acute virulence factors and reduced biofilm formation (Almblad et al., 2015). Accordingly,  $\text{HCO}_3^-$  stimulates soluble adenylate cyclase (sAC) and increases the activity of phosphodiesterase that breaks down c-di-GMP (Chen et al., 2000; Koestler and Waters, 2014a). Of note is that  $\text{HCO}_3^-$ ,  $\text{CO}_2$ , and external pH affect soluble adenylate cyclase activity and consequently intracellular cAMP levels (Hammer et al., 2006), which is a hallmark for *Pseudomonas aeruginosa* virulence factors that decrease when bacteria form biofilms in chronic infection (Valentini and Filloux, 2016). Thus, bacterial life style (planktonic vs. biofilm) is, at least partially, dictated by intracellular cAMP levels.

Herein, we focus on the effects of  $\text{HCO}_3^-$  on planktonic growth on several pathogens common to cystic fibrosis and consider biofilm forming capacity for two of the more prevalent bacteria in CF, *P. aeruginosa* and *S. aureus*. This work presents a novel proposal that  $\text{HCO}_3^-$  may therapeutically help prevent colonization and biofilm formation of CF-associated bacteria by increasing intracellular cAMP levels. Not only do the data confirm earlier notions, but they also add further reasons to consider inhaled  $\text{HCO}_3^-$  as a potential therapy, especially in the context of CF where defective  $\text{HCO}_3^-$  secretion is a well-established basic defect.

## MATERIALS AND METHODS

### Growth Experiments

The growth of ATCC control strains and clinical isolates of different Gram-positive and Gram-negative bacteria in Brain-Heart Infusion (BHI) broth (Mast Group Ltd., Merseyside, United Kingdom) were compared with and without 100 mmol  $\text{l}^{-1}$   $\text{NaHCO}_3$  in the medium and equilibrated with  $\text{CO}_2$  to control pH. Growth experiments were carried out with the following reference strains: *S. aureus* ATCC 25923 (MSSA), *S. aureus* ATCC 29213 (MSSA), *S. aureus* ATCC 33591 (MRSA), *Streptococcus agalactiae* ATCC 80200, *Enterococcus faecalis* ATCC 29212, *E. faecalis*, vanB+, ATCC 51299, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *Haemophilus influenzae* ATCC 49766, *H. influenzae* ATCC 49247. Some of the experiments were repeated with clinical isolates of the same species, obtained from the Central Bacteriological Diagnostic Laboratory of Semmelweis University, Budapest, obtained from daily routine specimens.

The density of bacterial suspensions was set at 0.5 McFarland (approximately  $10^8$  CFU  $\text{ml}^{-1}$ ) with a VITEK Densichek apparatus (Biomérieux, Marcy l'Etoile, France). The pH of the 100 mmol  $\text{l}^{-1}$   $\text{NaHCO}_3$ -containing BHI broth was set at pH approximately 7.4 by bubbling the autoclaved solution to equilibration with 20%  $\text{CO}_2$ -balance air for at least 16 h at 37°C before inoculation. The pH of these unstirred solutions in hermetically capped bottles remained stable for at least 24 h. To achieve other pH values, the  $\text{CO}_2$  concentration required was calculated from the Henderson-Hasselbalch equation for the  $\text{HCO}_3^-/\text{CO}_2$  buffer system (Story, 2004). When BHI broth containing 100 mmol  $\text{l}^{-1}$   $\text{NaHCO}_3$  was equilibrated with 5%  $\text{CO}_2$ , the measured pH ( $\sim 8.5$ ) was somewhat higher than that calculated from the Henderson-Hasselbalch equation (pH  $\sim 8.0$ ).

Aliquots of each suspension (200  $\mu\text{l}$ ) were dispensed into 96-well microtiter plates in duplicate. Bacterial suspensions were then incubated in ambient air ( $\sim 0.04\%$   $\text{CO}_2$ ), in 5% or in 20%  $\text{CO}_2$  by design. Bacterial growth was followed by measuring the optical density (OD) at 595 nm using a PR2100 microplate reader (Bio-Rad Laboratories, Hercules, Canada) 60 min after inoculating and subsequently every 15 min for 5.5 h. Optical density of a negative control (without bacterial growth) was subtracted from all OD values. The results of the parallel measurements of duplicate samples were averaged and normalized to the control media. The growth rates were determined by calculating the area under the curve (AUC)

(Horváth et al., 2012) using Microsoft Excel, based on the summation of small trapezoids.

The osmolality of BHI broth was approximately 360 mosm  $\text{kg}^{-1}$  in accordance with the data published earlier (Montgomerie et al., 1972). Since supplementation of BHI broth with 100 mmol  $\text{l}^{-1}$   $\text{NaHCO}_3$  increased the osmolality of the solution to approximately 470 mosm  $\text{kg}^{-1}$ , bacterial growth was also determined in BHI broth containing 100 mmol  $\text{l}^{-1}$   $\text{NaCl}$  (pH 7.4 adjusted with  $\text{NaOH}$ ) as a control. As a pH control, the growth of bacteria was measured in unsupplemented BHI broths adjusted with  $\text{HCl}$  or  $\text{NaOH}$  to pH values ranging from 6.8 to 9.0 as required.

### Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) by Broth Microdilution

To determine MIC and MBC, two strains of *P. aeruginosa* (ATCC 27853 and a clinical isolate) and two strains of *S. aureus* (ATCC 29213 and a clinical isolate) were selected. Bacteria were cultured for 18–20 hours on blood agar plates. The following day, a 0.5 McFarland suspension was prepared in physiological salt solution. This suspension was diluted 1:20 in Mueller–Hinton (MH) Broth (cation adjusted). For further measurements this standardized inoculum was used.  $\text{NaHCO}_3$  was diluted serially twofold in MH Broth (cation adjusted) in a plastic microdilution plate with round bottom wells to a final volume of 0.1 ml. The concentration of  $\text{NaHCO}_3$  varied from 1000 to 1.95 mmol  $\text{l}^{-1}$  in 10 steps of twofold dilutions. The wells were inoculated with 0.01 ml of standardized inoculum, positive and negative growth controls were also used. After 24 h incubation, the growth of bacteria was evaluated based on the visible change of turbidity. MIC was identified as the lowest concentration of  $\text{HCO}_3^-$  at which no visible growth was observed. To determine MBC, specimens from the wells without visible bacterial growth were inoculated onto antibiotic-free agar plates and incubated for 24 h. MBC was defined as the lowest concentration of  $\text{HCO}_3^-$  where no colonies were observed.

### Biofilm Experiments

Although almost all bacteria involved in CF lung disease can form biofilms, *P. aeruginosa* presents the largest clinical challenge. Therefore, we investigated *P. aeruginosa* for biofilm formation. Isolates were grown overnight to stationary phase in bouillon containing meat extract 0.3%, yeast extract 0.2%, pepton 1%,  $\text{NaCl}$  0.5%; pH adjusted to 7.5 with  $\text{NaOH}$ . The overnight cultures were diluted 1:100 in the desired medium. All solutions were prepared by filtration using 0.22  $\mu\text{m}$  filter membranes. Aliquots of 100  $\mu\text{l}$  of diluted cultures were pipetted into eight parallel wells of a 96-well microtiter plate. The covered plates were incubated at 37°C in ambient air, 5%, or 20%  $\text{CO}_2$  for 48 h. Planktonic bacteria were then removed by rigorous washing with PBS three times. Bacteria attached to the wells were air-dried and subsequently stained with 125  $\mu\text{l}$  of 0.1% crystal violet solution for 10 min. Excess crystal violet was removed by water-washing; that is, submerging the plates in tap water several times.

After air-drying, crystal violet was solubilized in 30% acetic acid (200  $\mu\text{l}$  per well) for 10 min. From each well, 125  $\mu\text{l}$  of this solution was then transferred to separate wells of an optically clear, flat-bottom 96-well plate. Optical density was measured at 595 nm in a PR2100 microplate reader (see above) (Merritt et al., 2011).

To measure the influence of glucose-content on biofilm formation, the bouillon was supplemented with 0.2, 1.0, 2.0, and 4.0 g  $\text{l}^{-1}$  glucose. Biofilm formation was absent at low glucose content and was the strongest at 4.0 g  $\text{l}^{-1}$  glucose, consequently all experiments were performed using bouillon containing 4.0 g  $\text{l}^{-1}$  glucose.

### Measurement of Intracellular cAMP Levels

The bacterial production of cAMP was determined with the Cyclic AMP XP® Assay Kit (Cell Signaling Technology, Leiden, Netherlands, originally designed for eukaryotic cells, but applicable for bacteria as well). Bacterial cultures were first grown in the desired medium for 15–16 h to stationary phase for conditioning, then diluted 1:500 in 10 ml of the same fresh medium and allowed to grow for a few hours until reaching log phase, that is,  $\text{OD}_{595} = 0.4$ – $0.5$ . Cultures were then centrifuged (4000  $\times$  g, 5 min), bacteria were re-suspended in 200  $\mu\text{l}$  of the kit lysis buffer for 30 min on ice and centrifuged again (8000  $\times$  g, 5 min); 50  $\mu\text{l}$  of the supernatant was transferred to the assay plate. According to kit protocol, absorbance was measured at 450 nm in a PR2100 microplate reader.

### Statistical Analysis

For statistical analysis Statistica for Windows 7.0 (Statsoft) was used. Data presented are means  $\pm$  SD, if not indicated otherwise. The values were compared using ANOVA followed by LSD *post hoc* comparison test. Changes were considered statistically significant at  $P < 0.05$ .

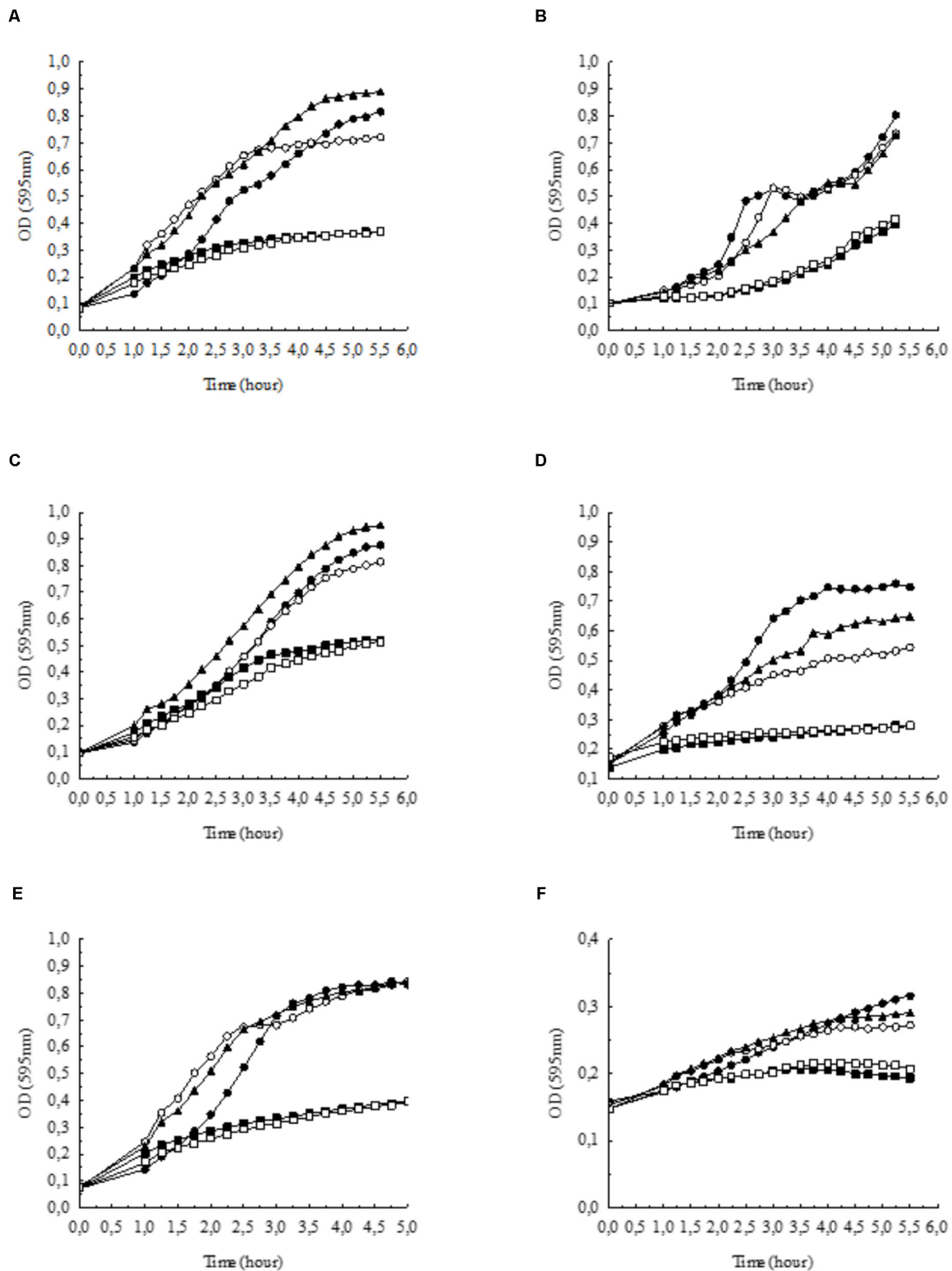
## RESULTS

### Reduction of Bacterial Growth by Bicarbonate

Since high external  $\text{HCO}_3^-$  concentrations and/or alkaline pH are reported to inhibit the growth of *E. coli* (Xie et al., 2010), we tested the effect of  $\text{HCO}_3^-$  on the growth of *E. coli*. The growth rate of bacteria was significantly inhibited in BHI broth supplemented with 100 mmol  $\text{l}^{-1}$   $\text{NaHCO}_3$  (pH 7.4, equilibrated with 20%  $\text{CO}_2$ ) as compared to control media (pH 7.4) without added  $\text{HCO}_3^-$  (Figure 1A). We tested the growth of bacteria in BHI broth supplemented with 100 mmol  $\text{l}^{-1}$   $\text{NaCl}$  (pH 7.4;  $\sim \Delta 110$  mosm  $\text{kg}^{-1}$ ), which did not suppress the growth of bacteria, indicating that the inhibitory effect of  $\text{NaHCO}_3$  was not due to increased osmolality or ionic strength.

Next, we tested whether alkaline pH affects bacterial growth. In BHI broth at pH 8.5, the growth capacity was not significantly influenced (Figure 1A); however, when bacteria were incubated





**FIGURE 1 |** Growth of (A) *Escherichia coli*, (B) *Pseudomonas aeruginosa*, (C) *Staphylococcus aureus*, (D) *Streptococcus agalactiae*, (E) *Enterococcus faecalis*, and (F) *Haemophilus influenzae* in BHI broth supplemented with  $\text{NaHCO}_3$  compared to control conditions (●: control pH = 7.4, ▲: 100 mmol  $\text{l}^{-1}$  NaCl, pH = 7.4). Bicarbonate-containing solutions were equilibrated with either 5% (□: 100 mmol  $\text{l}^{-1}$   $\text{NaHCO}_3$ , pH = 8.5) or 20%  $\text{CO}_2$  (■: 100 mmol  $\text{l}^{-1}$   $\text{NaHCO}_3$ , pH = 7.4). In the absence of bicarbonate, pH was adjusted to 8.5 with NaOH (○: growth at pH = 8.5). Each curve shows the average of two parallel experiments. Standard Deviations were generally less than 1% of the mean and are not shown. Note that in panel (F) the scale on the y axis differs from the other panels.

**TABLE 1** | Calculated AUC values of different bacteria, based on growth curves in **Figures 1A–F**.

Bacterium	Growth control pH = 7.4	Growth at pH = 8.5	100 mM NaCl pH = 7.4	100 mM NaHCO <sub>3</sub> pH = 7.4	100 mM NaHCO <sub>3</sub> pH = 8.5
<i>E. coli</i>	2.45	2.82	3.04	1.57	1.49
<i>P. aeruginosa</i>	2.05	1.92	1.82	1.01	1.06
<i>S. aureus</i>	2.48	2.41	2.93	1.92	1.76
<i>S. agalactiae</i>	2.84	2.20	2.48	1.27	1.35
<i>E. faecalis</i>	2.90	3.21	3.18	1.64	1.54
<i>H. influenzae</i>	1.28	1.26	1.31	1.05	1.07

in BHI broth supplemented with 100 mmol l<sup>-1</sup> NaHCO<sub>3</sub> at pH 8.5 (5% CO<sub>2</sub>), the inhibitory effects were similar to those observed at pH 7.4 (20% CO<sub>2</sub>) (**Figure 1A**). Thus, the data show that NaHCO<sub>3</sub> *per se* inhibited bacterial growth, which was not simply due to higher osmolality or alkaline media.

In order to test whether the inhibitory effect of HCO<sub>3</sub><sup>-</sup> is specific to *E. coli* cells, we investigated other species such as *P. aeruginosa*, *S. aureus*, *S. agalactiae*, *E. faecalis*, and *H. influenzae*. Similarly to the effects on *E. coli*, NaHCO<sub>3</sub> (100 mmol l<sup>-1</sup>) significantly inhibited the growth of all these species as well, suggesting that HCO<sub>3</sub><sup>-</sup> can suppress bacterial growth in general (**Figures 1B–F**). In order to compare the growth rates of bacteria under different conditions more quantitatively, AUC values were determined as a measure of the reduced growth rates in HCO<sub>3</sub><sup>-</sup>-enriched medium for each bacterial specie (**Table 1**).

In the previous experiments, to maintain the initial pH of the media near 7.4, all bicarbonate-containing solutions were equilibrated with calculated levels of CO<sub>2</sub>. Therefore, we asked the question whether HCO<sub>3</sub><sup>-</sup>-containing media without CO<sub>2</sub> equilibration influences growth of *P. aeruginosa*. Despite the fact that media pH continued to increase during the exponential growth phase (i.e., the first six hours), bacterial density remained similar to those observed with CO<sub>2</sub> equilibration (**Figures 2A,B**). When bacteria were grown until reaching the stationary phase (i.e., 24 h), significant increases in OD values were observed in the presence of both 25 and 100 mmol l<sup>-1</sup> HCO<sub>3</sub><sup>-</sup> suggesting that HCO<sub>3</sub><sup>-</sup> is bacteriostatic rather than bactericidal. Of note is that a lower bacterial density was observed at 24 h when 100 mmol l<sup>-1</sup> HCO<sub>3</sub><sup>-</sup> was present, but it should be kept in mind that excessive alkalization at atmospheric CO<sub>2</sub> levels, may exert inhibitory effects on growth rate (**Figure 2A**). Nonetheless, HCO<sub>3</sub><sup>-</sup>-containing media of concentrations up to 100 mmol l<sup>-1</sup> equilibrated with appropriate levels of CO<sub>2</sub> did not inhibit maximal bacterial growth at 24 h, suggesting that the bacteriostatic effects of HCO<sub>3</sub><sup>-</sup> occur within the initial period of the exponential growth phase (**Figure 2B**). We obtained similar results when the above experiments were repeated with *S. aureus*, at least with respect to bacterial growth profile (**Figures 3A,B**).

## Results of the MIC and MBC Determination

In the case of all four tested isolates, an MIC of 125 mmol l<sup>-1</sup> was obtained. The MBCs for the two *P. aeruginosa* strains

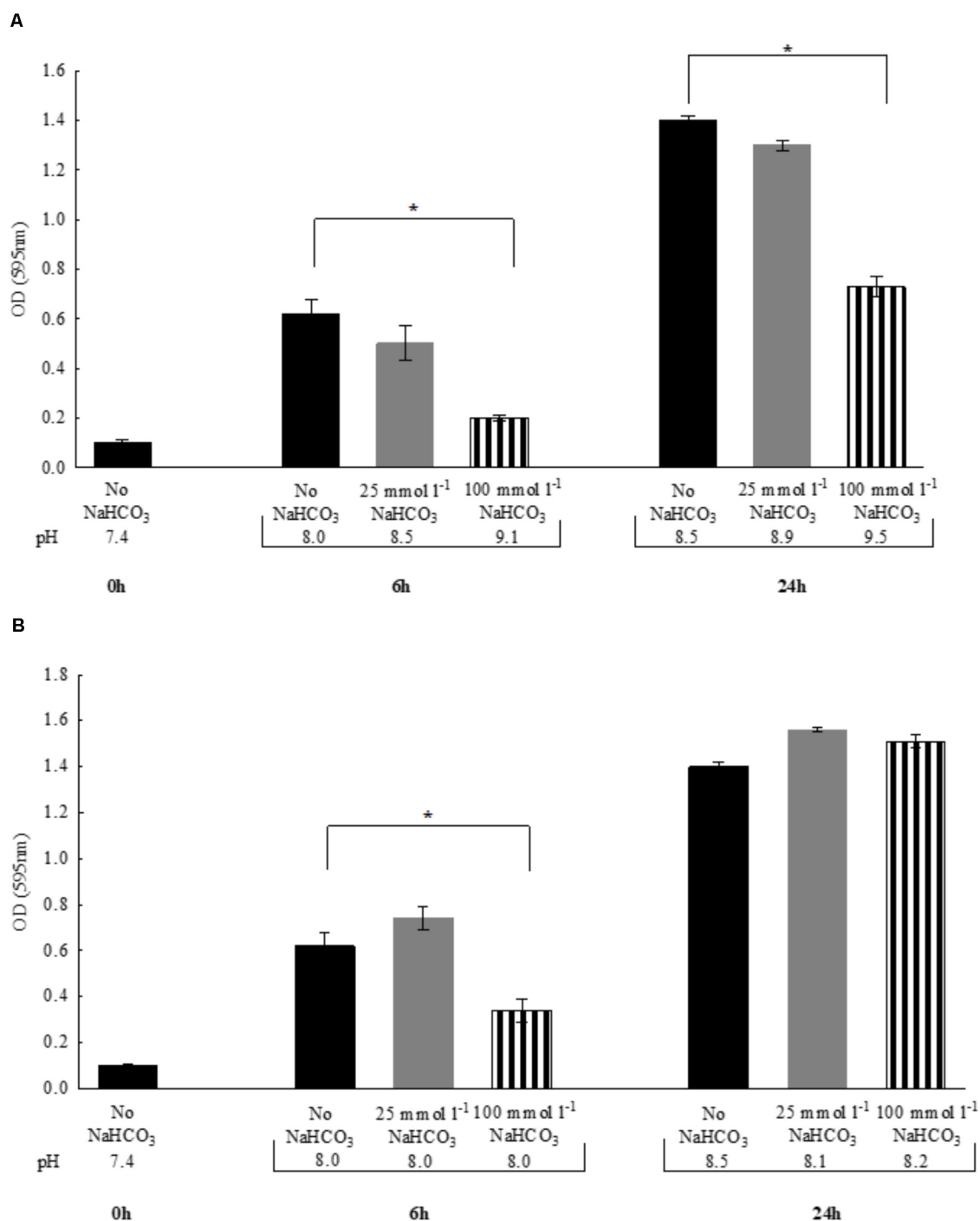
were 500 mmol l<sup>-1</sup>, meanwhile the two *S. aureus* isolates remained alive even in the highest HCO<sub>3</sub><sup>-</sup> concentration tested (MBC > 1000 mmol l<sup>-1</sup>).

## Bicarbonate Inhibits Biofilm Formation of *P. aeruginosa*

One of the most severe complications of CF lung disease involves biofilm formation of pathogenic bacteria, which may be due to depleted levels of HCO<sub>3</sub><sup>-</sup> in CF airways. Thus, we asked the question whether HCO<sub>3</sub><sup>-</sup> could not only inhibit planktonic bacterial growth, but biofilm formation as well. Glucose starvation leads to impaired biofilm formation associated with elevated cAMP levels (Huynh et al., 2012), hence glucose is required for optimal biofilm formation. Similarly, our data show that although bacteria achieved high density (OD<sub>595</sub> = 0.94 ± 0.12, *n* = 7), no biofilm was detected in the medium without added glucose (**Figure 4**). On the other hand, in the presence of glucose, bacterial growth was similar (OD<sub>595</sub> = 0.81 ± 0.06, *n* = 7) and robust biofilm formation was observed. However, the supplementation of glucose-containing medium with 100 mmol l<sup>-1</sup> HCO<sub>3</sub><sup>-</sup> prevented biofilm formation and significantly inhibited planktonic growth as well (OD<sub>595</sub> = 0.45 ± 0.04, *n* = 8). To demonstrate the reduced number of viable cells in HCO<sub>3</sub><sup>-</sup>-containing media, a bacteria count was performed after 48 h' incubation. In the absence of HCO<sub>3</sub><sup>-</sup>, the calculated CFU in bouillon with and without glucose was 2.8 × 10<sup>12</sup> and 4.5 × 10<sup>11</sup>, respectively. In contrast, the average CFU ml<sup>-1</sup> was 6.6 × 10<sup>6</sup> in media supplemented with 100 mmol l<sup>-1</sup> HCO<sub>3</sub><sup>-</sup>. In addition, 50 mmol l<sup>-1</sup> HCO<sub>3</sub><sup>-</sup> partially blocked biofilm formation suggesting a concentration-dependent inhibitory mechanism (**Figure 4**). When NaCl (100 mmol l<sup>-1</sup>) replaced NaHCO<sub>3</sub>, biofilm formation capacity was fully maintained.

## Bicarbonate Increases Intracellular cAMP Levels in Bacteria

Inhibition of biofilm formation may be induced by HCO<sub>3</sub><sup>-</sup> effects on the levels of cAMP and c-di-GMP. Thus, we investigated the effects of HCO<sub>3</sub><sup>-</sup> on intracellular cAMP production in *P. aeruginosa*. Supplementation of BHI medium with 25 and 100 mmol l<sup>-1</sup> HCO<sub>3</sub><sup>-</sup> resulted in significant concentration-dependent increases in cAMP levels (**Figure 5A**). Importantly, administration of 100 mmol l<sup>-1</sup> NaCl did not influence cAMP concentrations (**Figure 5A**). Since it is well known that sAC

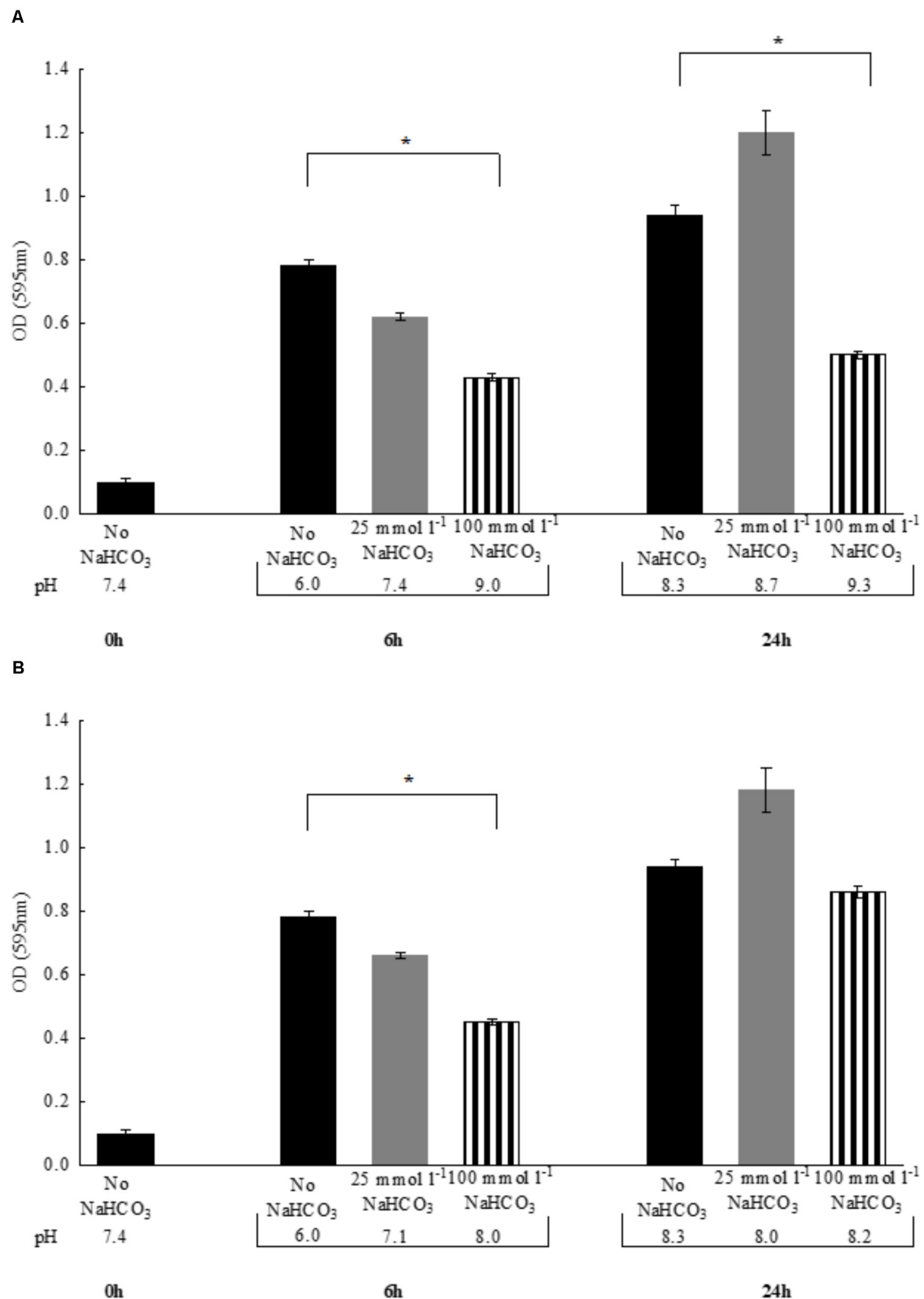


**FIGURE 2 |** Changes in bacterial density of *Pseudomonas aeruginosa* and media pH following 6 vs. 24 h incubations either **(A)** in air atmosphere, or **(B)** in the presence of appropriate levels of CO<sub>2</sub>. Black bars: NaHCO<sub>3</sub>-free controls, gray bars: 25 mmol l<sup>-1</sup> NaHCO<sub>3</sub>; striped bars: 100 mmol l<sup>-1</sup> NaHCO<sub>3</sub>. Statistically significant, \**P* < 0.05.

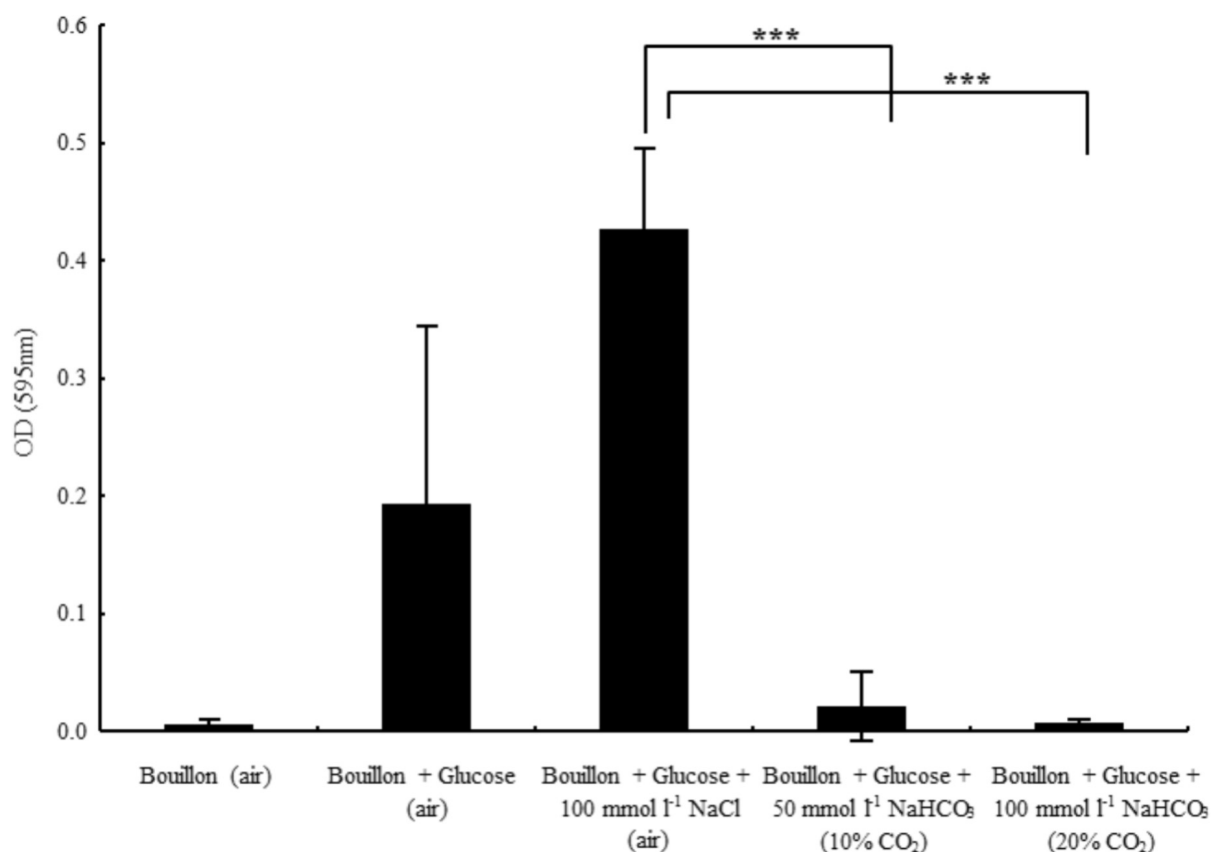
activity depends on intracellular pH (Rahman et al., 2013), we tested the effects of media pH changes between 6.0 and 9.0 on cAMP production. In this pH range, a slight increase in cAMP levels, parallel to alkalinization was observed (**Figure 5B**). Of note, even the highest pH-induced increase in cAMP level was

significantly lower than the increase induced by 100 mmol l<sup>-1</sup> HCO<sub>3</sub><sup>-</sup>. Similar results were obtained when the experiments were repeated with *S. aureus* (**Figure 5A**). Taken together, the data suggest that biofilm suppression by HCO<sub>3</sub><sup>-</sup> is mediated through an increased production of intracellular cAMP.





**FIGURE 3 |** Changes in bacterial density of *Staphylococcus aureus* and media pH following 6 vs. 24 h incubations either **(A)** in air atmosphere or **(B)** in the presence of appropriate CO<sub>2</sub> levels. Black bars: NaHCO<sub>3</sub>-free controls, gray bars: 25 mmol l<sup>-1</sup> NaHCO<sub>3</sub>, striped bars: 100 mmol l<sup>-1</sup> NaHCO<sub>3</sub>. \**P* < 0.05.



**FIGURE 4 |** Biofilm formation capacity of *Pseudomonas aeruginosa* in glucose-containing bouillon (4 g l<sup>-1</sup>) in the presence of sodium chloride and two different concentrations of sodium bicarbonate. Please note that in the absence of glucose no biofilm formation was observed. \*\*\**P* < 0.001.

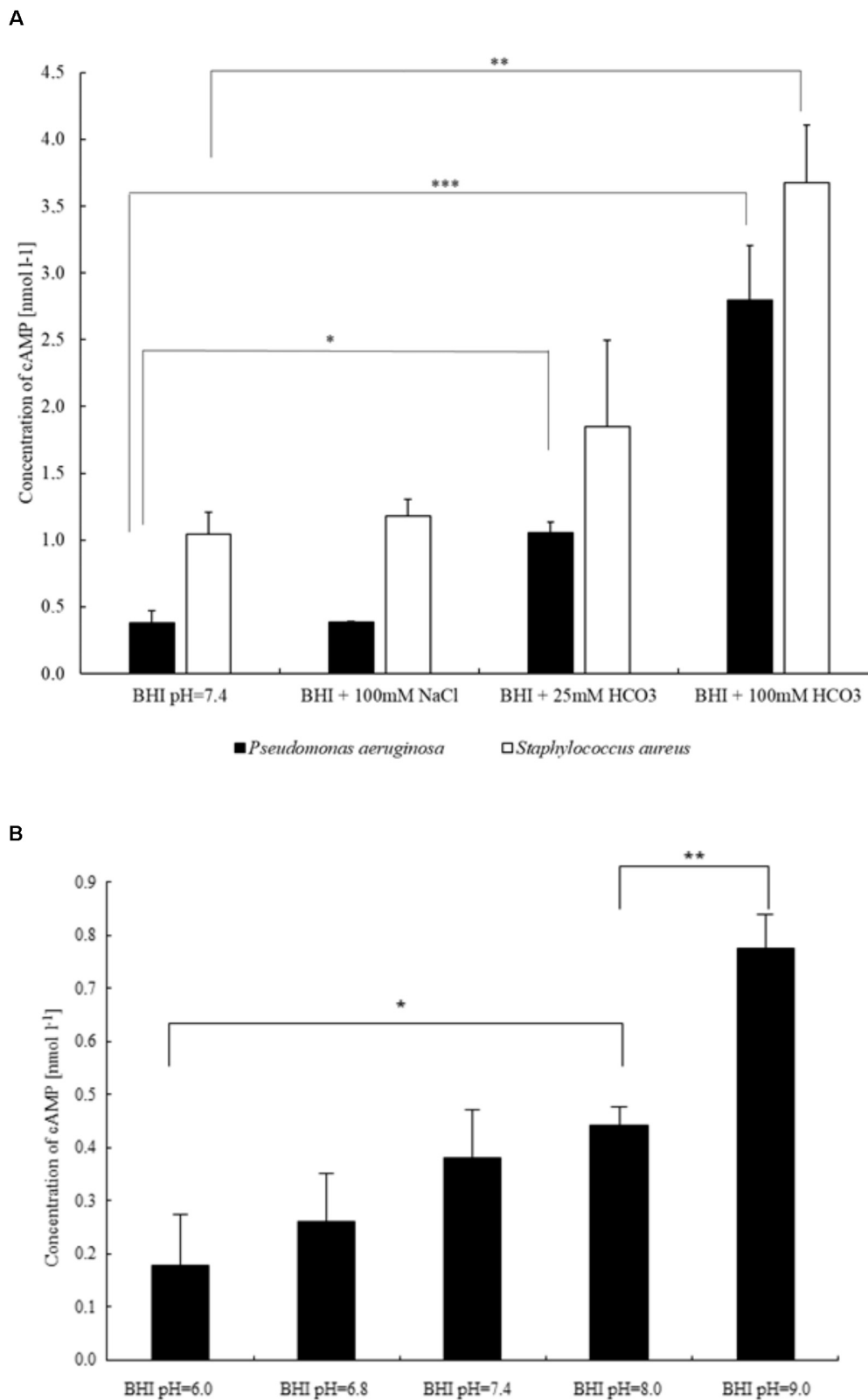
## DISCUSSION

Early studies using the human serous cell line (Calu-3) showed that airway-derived epithelial cells can secrete HCO<sub>3</sub><sup>-</sup> (Lee et al., 1998). Recently, active HCO<sub>3</sub><sup>-</sup> secretion was demonstrated in native intact small airway epithelium as well (Shamsuddin and Quinton, 2014). Loss of HCO<sub>3</sub><sup>-</sup> secretion seems to be associated with different pathological consequences in CF airways, such as formation of thickened mucus (Quinton, 2010), reduced microbial susceptibility to antimicrobial peptides (Dorschner et al., 2006) and impaired bacterial killing capacity (Pezzulo et al., 2012; Abou Alaiwa et al., 2014). Therefore, the delivery of HCO<sub>3</sub><sup>-</sup> into the airways may be potentially therapeutic in CF (Pezzulo et al., 2012; Li et al., 2016). Moreover, beyond CF, decreased pH (likely due to decreased HCO<sub>3</sub><sup>-</sup>) of airway surface liquid and chronic bacterial infections have also been described in other chronic airway diseases such as COPD (Mall and Hartl, 2014; Li et al., 2016; Shah et al., 2016a,b).

A shift to either acidic or alkaline external pH presents a stress for bacteria, which may influence survival and growth (Padan et al., 2005). In alkaline environments the growth rate of neutrophilic bacteria is reduced (Maurer et al., 2005). When grown in media at pH 7.0, *E. coli* cells exhibit shorter generation time compared to that when it is cultured at pH

8.7 (Maurer et al., 2005). Our data show that 100 mmol l<sup>-1</sup> NaHCO<sub>3</sub> significantly inhibit the growth of *E. coli* equally, at both pH 7.4 and 8.5, but in the absence of HCO<sub>3</sub><sup>-</sup>, neither alkaline pH (up to 8.5) nor equivalent increases in osmolality (NaCl) inhibit bacterial growth, indicating that HCO<sub>3</sub><sup>-</sup> *per se* plays a pivotal role in growth suppression. Alkaline conditions may alter cytosolic pH of bacteria when protons must be taken up from the extracellular medium. Under alkaline conditions proton scavengers markedly decrease *E. coli* viability (Vanhautegehem et al., 2013). This phenomenon can be explained by partitioning of unprotonated scavengers into the cytosol where they become protonated and increase cytosolic pH. To maintain pH homeostasis, bacteria require high ATP consumption and membrane hyperpolarization. We surmise that higher HCO<sub>3</sub><sup>-</sup> concentrations in the media increase the gradient for increased HCO<sub>3</sub><sup>-</sup> entry into the cytosol of bacteria elevating intracellular pH. The higher energy consumption occurs at the expense of bacterial growth rate. Importantly, similar results were observed with *S. aureus*, *P. aeruginosa*, *S. agalactiae*, *E. faecalis*, and *H. influenzae* as well, again suggesting that HCO<sub>3</sub><sup>-</sup> might be used to inhibit the growth of bacteria more generally.

*S. aureus* and *P. aeruginosa* are of particular interest because of their high incidence in CF patients with chronic pulmonary infections. We observed that during the first few hours of



**FIGURE 5 |** Differences in intracellular cAMP production of bacteria influenced by **(A)** sodium chloride and two different concentrations of sodium bicarbonate (*P. aeruginosa* and *S. aureus*), and **(B)** external pH (*P. aeruginosa*). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



growth *S. aureus* decreased, whereas *P. aeruginosa* increased the pH of the medium. These data suggest that  $\text{HCO}_3^-$ , rather than pH shifts, suppressed bacterial growth. In *P. aeruginosa* these observations were confirmed in  $\text{HCO}_3^-$ -containing media without  $\text{CO}_2$  equilibration where despite the increase in the pH of the media, the growth rate was similar to that detected with  $\text{CO}_2$  equilibration. Extending the incubation to 24 h clearly demonstrated that the effects of  $\text{HCO}_3^-$  were bacteriostatic rather than bactericidal. Furthermore, in the absence of  $\text{CO}_2$  a critically high pH ( $>9.2$ ) occurred with a reduced growth rate.

Recurrent lung infections caused by long-term colonization of biofilm-forming bacteria are a significant threat for CF patients (Høiby et al., 2017). Importantly,  $\text{NaHCO}_3$  disrupts oral biofilms *in vitro* (Pratten et al., 2016).  $\text{NaHCO}_3$  in combination with sodium metaperiodate and sodium dodecyl sulfate also suppressed the formation of *P. aeruginosa* biofilms (Gawande et al., 2008). We also confirmed these observations as  $\text{NaHCO}_3$  prevented biofilm formation at  $100 \text{ mmol l}^{-1}$  and inhibited the planktonic growth of bacteria. Since the administration of equimolar  $\text{NaCl}$  ( $100 \text{ mmol l}^{-1}$ ) had no effect and lower concentrations of  $\text{NaHCO}_3$  only partially blocked biofilm formation, we concluded that in our experimental model  $\text{HCO}_3^-$  suppresses bacterial conversion to biofilms as a function of concentration. These observations may be therapeutically promising as our unpublished data suggest that  $100 \text{ mmol l}^{-1}$   $\text{NaHCO}_3$  does not have toxic effects on airway epithelial cells *in vitro*.

Biofilm formation requires coordination between cAMP and c-di-GMP signaling in several bacteria. In *P. aeruginosa* increased levels of c-di-GMP suppress signaling from the cAMP-virulence factor regulator pathway and the expression of virulence factors that favor a persistent biofilm state (Almblad et al., 2015). On the other hand, cAMP stimulates the production of acute virulence factors, but inhibits biofilm formation in *Vibrio cholerae* (McDonough and Rodriguez, 2011). Likewise,  $\text{HCO}_3^-$  also activates virulence gene expression in *V. cholerae* (Iwanaga and Yamamoto, 1985; Abuaita and Withey, 2009). In the intestinal lumen,  $\text{HCO}_3^-$  apparently suppresses the bile-mediated induction of c-di-GMP that inhibits biofilm formation (Koestler and Waters, 2014b). Based on the above observations, we speculated that  $\text{HCO}_3^-$  should increase bacterial cAMP levels. In fact, our data show that  $\text{NaHCO}_3$  stimulates intracellular cAMP concentrations in both *P. aeruginosa* and *S. aureus*. The stimulatory effect was not observed in the presence of equivalent  $\text{NaCl}$  concentrations indicating a specific role for  $\text{HCO}_3^-$ . Interestingly, however, cAMP production was dependent on external pH in the range between 6.0 and 9.0. These results are consistent with the findings that sAC is regulated by various environmental signals such as calcium and  $\text{CO}_2/\text{HCO}_3^-/\text{pH}$  (McDonough and Rodriguez, 2011; Rahman et al., 2013). Chen et al. (2000) previously demonstrated that sAC functions as a  $\text{HCO}_3^-$  sensor in many biological systems. More recently,  $\text{HCO}_3^-$  has been shown to increase cAMP production via sAC stimulation in corals (Barott et al., 2013). Thus, we surmise that reduced  $\text{HCO}_3^-$  secretion from CF lung epithelial cells results in decreased luminal pH, which leads to decreased levels of cAMP production in bacterial cells. Low bacterial cAMP

levels reduce virulence factor production, and thus, fail to alert the innate host immune systems, allowing for more favorable conditions for biofilm formation. Parallel to these events, elevated concentrations of c-di-GMP may also support biofilm formation, making eradication of bacteria from the airways difficult.

## CONCLUSION

Since we found that increased  $\text{HCO}_3^-$  impedes the growth and biofilm formation of several pulmonary bacterial pathogens, we expect that increasing  $\text{HCO}_3^-$  in the airways may reduce infection, inflammation, and a consequent tissue damage in the lungs. We assume that the inhalation of aerosolized  $\text{HCO}_3^-$  could prove therapeutic against bacteria such as *S. aureus* and *P. aeruginosa*, which are among the most relevant pathogens in lung infections in CF. Although inhalation therapy is inherently episodic and therefore the concentration of  $\text{HCO}_3^-$  on the airways is certain to dissipate between inhalation intervals, the therapy may offer significant benefits even with only acute changes in the airway surface liquid composition by depressing bacterial growth repetitively and by increasing the ability of the innate immune system to reduce or clear the infection load. However, caution should be taken with treatments that may affect intracellular cAMP levels because increasing cAMP may enhance the expression of virulence factors and lead to acute exacerbation in CF patients with chronic bacterial infections (Coggan and Wolfgang, 2012). Routinely, CF patients are treated repeatedly with different courses of antibiotics in spite of growing concerns over antibiotic side effects. Although antagonistic effects of  $\text{HCO}_3^-$  have been reported for tobramycin efficacy (Kaushik et al., 2016), our unpublished preliminary data suggest that the efficacy of both erythromycin and imipenem increases in an alkaline environment. Based on these observations,  $\text{HCO}_3^-$ , inhaled regularly, may reduce use of antibiotics.

## AUTHOR CONTRIBUTIONS

OD, KL, BS, AK, BB, AT, SK, PJ, KR, and ÁZ were involved in lab experiments. PQ, ÁZ, and DO designed the study. ÁZ, DO, KL, and BS analyzed the data. ÁZ wrote the manuscript. PQ reviewed and critically revised the manuscript.

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## Review

## Extracellular ATP as a signaling molecule for epithelial cells

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**Abstract**

The charge of this invited review is to present a convincing case for the fact that cells release their ATP for physiological reasons. Many of our “purinergic” colleagues as well as ourselves have experienced resistance to this concept, because it is teleologically counter-intuitive. This review serves to integrate the three main tenets of extracellular ATP signaling: ATP release from cells, ATP receptors on cells, and ATP receptor-driven signaling within cells to affect cell or tissue physiology. First principles will be discussed in the Introduction concerning extracellular ATP signaling. All possible cellular mechanisms of ATP release will then be presented. Use of nucleotide and nucleoside scavengers as well as broad-specificity purinergic receptor antagonists will be presented as a method of detecting endogenous ATP release affecting a biological endpoint. Innovative methods of detecting released ATP by adapting luciferase detection reagents or by using “biosensors” will be presented.

Because our laboratory has been primarily interested in epithelial cell physiology and pathophysiology for several years, the role of extracellular ATP in regulation of epithelial cell function will be the focus of this review. For ATP release to be physiologically relevant, receptors for ATP are required at the cell surface. The families of P2Y G protein-coupled receptors and ATP-gated P2X receptor channels will be introduced. Particular attention will be paid to P2X receptor channels that mediate the fast actions of extracellular ATP signaling, much like neurotransmitter-gated channels versus metabotropic heptahelical neurotransmitter receptors that couple to G proteins. Finally, fascinating biological paradigms in which extracellular ATP signaling has been implicated will be highlighted. It is the goal of this review to convert and attract new scientists into the exploding field of extracellular nucleotide signaling and to convince the reader that extracellular ATP is indeed a signaling molecule.

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**Keywords:** ATP; Extracellular nucleotide signaling; P2X receptor channel**1. Introduction**

To understand autocrine and paracrine ATP signaling, the “first principles” of extracellular nucleotide and nucleoside signaling must be presented. No other review in the past spoke to all of the issues germane to extracellular ATP signaling than the review by Gordon in 1986 [1]. This review is modeled after that treatise, having benefited from 16 additional years of productive research as well as the emergence of new ideas. The first evidence that extracellular

adenine compounds may have physiological activities was described by Drury and Szent-Gyorgyi in 1929 [2]. This early study opened a new field of cardiovascular research investigating effects of extracellular purine nucleotides and nucleosides in blood flow. However, more than 40 years passed before adenosine re-emerged as an important autocrine and paracrine mediator in extracellular signaling [3]. In 1972, Burnstock [4] postulated that ATP could act as an extracellular signal in nerve-mediated responses of the smooth muscles in the gastrointestinal tract and bladder. Initially, this concept was met with considerable skepticism. Today, we already know that extracellular signaling through purinoceptors is vital not only in excitable cells and tissues but also in non-excitable cells and tissues.

Newly synthesized ATP is first made and transported out of the mitochondrion via oxidative phosphorylation. The

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steady-state cytosolic concentration of ATP is 3–10 mM, the fuel for countless metabolic and enzymatic reactions. Because ATP is essential for metabolism, release of this precious energy substrate is met with some anxiety. Nevertheless, if one assumes that the steady-state extracellular ATP is approximately 10 nM under basal conditions and intracellular ATP is 10 mM, the gradient for ATP secretion or efflux is approximately  $10^6$ -fold. This gradient is 100-fold greater, yet opposite to, the gradient for calcium entry into cells. Naturally, if a pathway is activated or opened for ATP release, ATP would exit the cell down a very favorable chemical concentration gradient. However, it must be emphasized that only 1% or less of the intracellular ATP pool needs to be released to activate maximally any and all receptors. Thus, accomplishment of extracellular ATP signaling can occur without compromising cellular metabolism or essential enzymatic reactions.

Purinoreceptors are divided into two classes: P1 or adenosine receptors and P2, which recognize primarily extracellular ATP, ADP, UTP and UDP [5]. The P2 receptors are further subdivided into two subclasses. P2X receptors are extracellular ATP-gated calcium-permeable non-selective cation channels that are modulated by extracellular  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{H}^+$  and metal ions, such as  $\text{Zn}^{2+}$  and/or  $\text{Cu}^{2+}$  [6]. P2Y receptors (with exception of P2Y<sub>12</sub> and P2Y<sub>13</sub>) couple to heterotrimeric G proteins and phospholipases (primarily phospholipase C $\beta$ ) to raise intracellular free calcium concentration [7]. Once released, ATP is free to bind to P2X and P2Y receptors, on the same cell or neighboring cells. Alternatively, ADP, a metabolite, may also bind with high affinity to a subset of P2 receptors. UTP and UDP follow

similar chemistry and have their specific P2Y receptor subtypes; however, the intracellular concentration of UTP is micromolar rather than millimolar. The final metabolite of ATP, adenosine, is also biologically active and binds to P1 receptors. Once these receptors are activated, signal transduction begins and affects cell or tissue function. These “first principles” are illustrated in Fig. 1 in the context of three neighboring epithelial cells in a polarized epithelium.

Once ATP leaves the cell, it is also degraded rapidly; thus, it is thought of as a local mediator that acts in an autocrine or paracrine manner within tissues and tissue microenvironments to stimulate its receptors before it is chemically modified. Ecto-enzymes secreted into the extracellular milieu as a secreted protein or membrane-bound as an ecto-enzyme target ATP. Ecto-apyrases cleave the gamma- and beta-phosphates of ATP, yielding ADP and 5'-AMP. Ecto-ATPases and ADPases also subserve this function. Ecto-5'-nucleotidases can convert 5'-AMP into adenosine, a higher-affinity purinergic agonist than ATP for its own class of P1 G protein-coupled receptors. Like any biochemical reaction, these can occur in reverse to re-synthesize ATP. Secreted or membrane-bound ecto-kinases such as adenylate kinase or nucleoside monophospho- and diphosphokinases can phosphorylate nucleosides to remake 5'-AMP, ADP and, ultimately, the triphosphate nucleotide ATP [8].

In this review, we put particular emphasis on mechanisms by which ATP is released from the cells and purinergic receptors and signaling in epithelial cells that transduce that extracellular ATP signal.

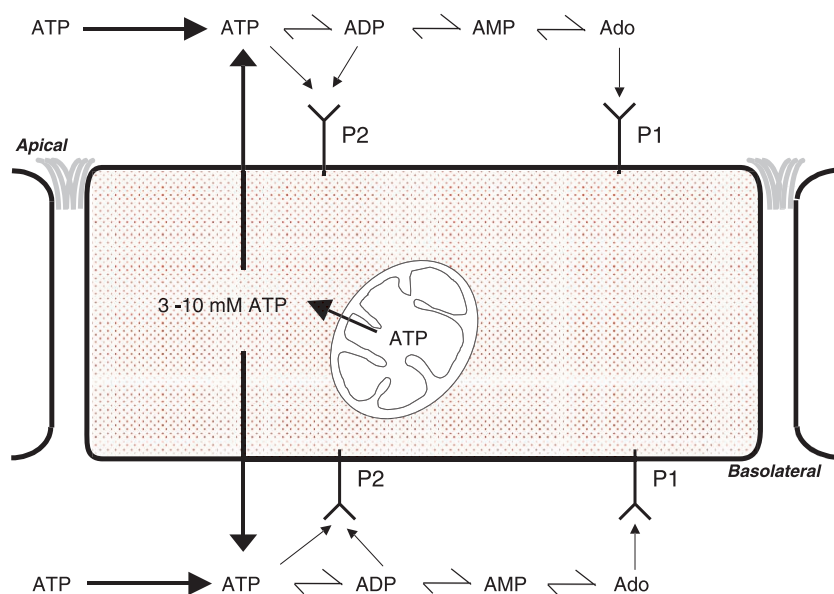


Fig. 1. The first principles of extracellular ATP signaling. This epithelial cell model illustrates the first principles of extracellular ATP signaling in the apical and basolateral spaces. Intracellular ATP generated by the mitochondria creates a large intracellular pool and an enormous gradient for ATP efflux, secretion, or release. Nevertheless, only 1% or less of this cytosolic ATP pool is required to be released to stimulate P2 receptors and stimulate signal transduction in the same cell or in a neighboring cell. Not every cell in the epithelium needs to release its ATP; it can diffuse from a neighboring cell that released it.

## 2. Mechanisms of ATP release into the extracellular milieu

Driven by the very favorable chemical concentration gradient for ATP secretion out of the cell, many possible ATP release mechanisms have been postulated. Considerable evidence has been amassed that adenosine is transported across the membrane by concentrative nucleoside transporters (CNTs) or equilibrative (bidirectional) nucleoside transporters (ENTs) [9]. By analogy, similar release mechanisms have been entertained for nucleotides. Emerging work has also shown that adenosine is also created in large part by extracellular metabolism of ATP. Lessons can first be derived from the mitochondrion. Newly synthesized ATP is transported across the inner or cristine membrane of the mitochondrion by an adenine nucleotide transporter (ANT) that exchanges an ATP molecule for its metabolite, ADP. Mitochondrial porins (also known as voltage-dependent anion channels (VDACs)) conduct the ATP across the outer membrane into the cytosol. There is considerable biological debate about which ATP transport mechanism is more critical for mitochondrial export.

### 2.1. ATP-permeable release channels

Pictured in the context of our favorite cell model (Fig. 2), the polarized epithelial cell, ATP could be conducted passively down its favorable chemical concentration gradient by ATP-permeable release channels. These are likely anion channels (plasma membrane forms of VDAC or other chloride channels) that have significant permeability to larger anions like gluconate and ATP. These ATP release channels could reside in the apical or basolateral membrane. The existence of ion channels, that are permeated by ATP anions, was underscored by many laboratories investigating whether the cystic fibrosis transmembrane conductance regulator (CFTR) conducted ATP<sup>-</sup> itself in addition to Cl<sup>-</sup> or regulated a separate ion channel that was permeable to ATP<sup>-</sup>. One group suggested that CFTR and the multidrug resistance transporter (mdr or P-glycoprotein) conducted ATP<sup>-</sup> [10,11]. Schwiebert et al. [12] showed that CFTR potentiated autocrine ATP release to upregulate a separate anion channel, the outwardly rectifying Cl<sup>-</sup> channel (ORCC). The simplest interpretation was that CFTR conducted ATP<sup>-</sup>; however, this group was careful to point out that CFTR, as a conductance regulator, might also regulate an ATP release channel, transporter, or other release pathway [12]. Subsequently, several other laboratories failed to show conduction of ATP<sup>-</sup> through CFTR [13–16]; each laboratory performed a limited number of experiments in their system of choice and stopped early in their study when the negative data mounted. Recent studies by Braunstein et al. [17] and by Sugita et al. [18] have shown that CFTR is likely not an ATP conductive channel itself; however, it is closely associated with a separate ATP-permeable channel that it regulates positively. Nevertheless, its membership in

the superfamily of ATP-binding cassette (ABC) transporters suggests that it could transport ATP and other larger anions non-conductively at larger rates, given the large range of substances transported promiscuously by ABC transporters.

A handful of laboratories have performed additional research to begin to identify ATP release pathways in cells, to understand why CFTR would potentiate ATP release from cells and in response to the best stimuli, and to identify more specifically any and all ATP-permeable release channels. An initial assumption is that ATP-permeable release channels are anion channels. ATP release assays in hepatocytes and cholangiocytes by Fitz et al., airway and renal epithelial cells by Schwiebert et al., and heterologous cells by both of these laboratories has identified the lanthanides, gadolinium and lanthanum, as blockers of ATP release under basal and stimulated conditions [17,19–21]. The lanthanides are known blockers of mechanosensitive ion channels that are permeable to cations as well as anions. Braunstein et al. [17] has also shown that the disulfonic stilbene, DIDS, is a blocker of ATP release that is even more potent than gadolinium. Taken together, blockade of ATP release in biological cells by these agents suggests that ATP release channels are important conduits for ATP release.

One assumption is that some specific anion channels may also be permeable to ATP<sup>-</sup>. Are there likely candidates for ATP-permeable anion channels? Thinnes et al. [22,23] among others have documented that plasma membrane forms of porins or voltage-dependent anion channels (PL-VDACs) exist in a variety of cell types. In one study, they show co-localization of CFTR with a PL-VDAC [22]. It is known that “maxi” Cl<sup>-</sup> channels (or large conductance anion channels) with voltage-dependent inactivation characteristics similar to VDAC have been found by numerous investigators in numerous cell types. In fact, Fitz et al. and Schwiebert et al. have studied such “maxi” anion channels in the past in Chinese hamster ovary (CHO) cells [24,25] and renal cortical collecting duct (CCD) cells [26,27], respectively. Because VDAC is a major conduit for release of newly synthesized ATP in the outer membrane of the mitochondrion [28], an ATP-permeable VDAC in the plasma membrane may be a major conductive pathway for ATP exit into the extracellular milieu. Light, Schwiebert and Stanton [26,27] showed that the “maxi” anion channel in RCCT-28A cells, an A-type intercalated cell line from rabbit CCD, had significant permeability to gluconate (Cl:gluconate 8:1) and bicarbonate (Cl:HCO<sub>3</sub> 2:1). Sabirov et al. [29] have shown recently that an ATP-permeable “maxi” anion channel is expressed in mouse mammary carcinoma (C127) cells. Recent preliminary work from Liu et al. [30] have implicated a “maxi” anion channel in ATP release from the macula densa induced by changes in NaCl concentration in the cTAL. It is postulated that this ATP-permeable “maxi” anion channel is responsible for ATP release from the macula densa that diffuses to and binds to P2 receptors in the glomerulus as an important signal for tubuloglomerular feedback, a form of single nephron “autoregulation.” A

similar maxi anion channel (approximately 380–400 pS) has been found in cell-attached and excised patch-clamp recordings from non-CF and CF airway epithelial primary cultures and cell lines and is permeable to a multitude of different anions including ATP (E.M. Schwiebert, unpublished observations). Taken together, it is clear that the VDAC-like “maxi” anion channels are a leading candidate for ATP release channels in the plasma membrane of many cell types and tissues.

ATP-permeable anion channels may not be limited to “maxi” anion channels. At least two other candidates have been implicated indirectly in published studies. These include, but are not limited to, the ORCC and the volume-sensitive organic anion channel (VSOAC). In a study by Braunstein et al. [17], planar lipid bilayer experiments where bovine tracheal epithelial vesicle protein was fused with the bilayers containing or immunodepleted of CFTR protein. In each condition, an ATP conductance of approximately 20 pS was recorded that was potentiated by hydrostatic pressure applied to the bilayer and was inhibited by DIDS, gadolinium, and DPC. In the condition immunodepleted of CFTR protein, the ORCC was still present, as determined by removing ATP-containing solutions and replacing ATP with  $\text{Cl}^-$  containing solutions. The simplest interpretation was that the 40–80 pS ORCC  $\text{Cl}^-$  channel had a partial permeability to ATP. Because ORCCs may be conferred by CLC-3 and/or CLC-5 channels (or a heteromeric mixture of these two CLC channels along with additional CLC subtypes), CLC channels may also be candidates for ATP release channels. Another, equally valid, interpretation was that the 20 pS ATP channel was conferred by a separate protein. Another candidate for this ATP channel is the VSOAC channel first described and named as such by Strange et al. In an initial characterization, Jackson and Strange [31] showed that intracellular mM ATP concentrations supported VSOAC currents, while supraphysiologic external concentrations of mM ATP actually blocked macroscopic VSOAC currents carried by  $\text{Cl}^-$  or by organic osmolytes such as betaine or taurine. Because this external block was concentration- and voltage-dependent, ATP can be deduced to be a possible pore blocker of VSOAC and, thus, a partially permeable species along with large anions such as taurine and betaine. It has been shown that non-excitable cells, like hepatocytes, communicate via nucleotide release [32]. ATP efflux is not uniform across a field of cells but is restricted to abrupt point-source bursts [33]. Transient activation of connexin hemichannels is thought to mediate this ATP release [34].

It is important to note that these release channels may differ in their expression as well as their polarity of expression (if studied, in epithelial or endothelial cells or in neurons). The next few years of work by several laboratories should uncover much new information in the arena of ATP release channels. Arguably, this field was brought into the light via the controversy concerning CFTR, mdr, and ATP permeability through these ABC transporters.

Without that series of papers for and against this phenomenon, it is possible that this field may not have emerged this rapidly or at all.

## 2.2. Adenine nucleotide transporters

Like the nucleoside transporters, concentrative or equilibrative nucleotide transporters may also exist for ATP that are carriers or permeases. The ABC transporters such as the CFTR, the multidrug resistance proteins (mdr, MRP), and the multiple organic anion transporters (MOATs) may act as non-conductive transporters of ATP, along with a multitude of other substrates. Exchangers may exist for ATP in the plasma membrane, as they do in mitochondrial inner membrane, where ADP may be the exchanged substrate. Other substrates such as  $\text{Na}^+$  or  $\text{Cl}^-$ , which have favorable entry gradients, may be exchanged for ATP. This could also occur apically or basolaterally. These possible transport mechanisms are also illustrated in Fig. 2.

Even though evidence is emerging that ABC transporters do not conduct ATP at rates consistent with an ionic channel, these transporters may transport ATP across membranes at rates that may lie in a range more consistent with a transporter. The fact that glibenclamide, tamoxifen, cyclosporin A, and verapamil inhibit ATP release in several systems underscores this possibility [35]. Indeed, Linsdell and Hanrahan [36] have shown that CFTR transports glutathione at rates that border channel conduction rates ( $10^7$  ions/s and above) versus transporter rates ( $10^6$  molecules/s or below). In fact, symport of glutathione and ATP or ATP with another substrate could occur. KCl symporters are critical in red blood cell and hematopoietic cell volume regulation [37]; a KATP symporter is also not out of the realm of possibility. Moreover, the promiscuity of ABC transporters (mdr, MRP) and the substrates that they move across membranes suggests that ATP could be carried along for the ride with other countless substrates [35]. MOATs are also possible candidates [35,38]. Investigators should keep an open mind to this possible mechanism of ATP release.

As with VDAC or porin channels in outer mitochondrial membrane, ANTs that transport newly synthesized ATP across inner mitochondrial membrane to the exchange of ADP, also play a critical role in intracellular ATP transport [39]. Once thought to be restricted to the mitochondrion, emerging evidence suggests that ANTs may also be expressed in endoplasmic reticulum, the nuclear envelope membrane, and in brain synaptic vesicles [40–42]. The latter result suggests that ANTs may reside, at least for a brief period of time, in the plasma membrane of neurons once synaptic vesicles fuse to release their contents. A reason for addressing possible expression in synaptic vesicles is the known fact that ATP is present in high (millimolar) concentrations along with neurotransmitters or neuroendocrine agonists such as histamine and epinephrine. Such exchange of ATP released into the extracellular milieu with a metabolite of ATP transported back into the cell for



re-synthesis to ATP is also a viable hypothesis. Atractyloside, a specific inhibitor of the ANTs, can be used as an extracellular inhibitor of ATP release to probe the role of ANTs in autocrine ATP release and signaling.

### 2.3. ATP-filled vesicle

ATP-filled vesicles, which may also contain additional agonists or co-agonists, may fuse with the plasma membrane releasing ATP. It is a known fact, dating back to the work of Gordon [1] and Burnstock [4] and their seminal reviews on the topic of extracellular ATP or “purinergic” signaling, that a high content of ATP is found in synaptic vesicles with other neurotransmitters, mast cell granules with histamine, and chromaffin granules with epinephrine. ATP and its metabolites are known co-transmitters that modulate the effect of excitatory or inhibitory neurotransmitters. ADP and ATP are also released by platelets on their own via exocytosis for a “self-aggregation” signal at the clotting zone. Release of ATP-filled vesicles is the principal transmitter signal for “purinergic” nerves in the gut, the nociceptive nodose neurons in the dorsal root ganglia of the spinal cord, and in specialized nerves in the brain. Thus, exocytosis of ATP-filled vesicles, preformed and poised to fuse beneath the plasma membrane, should be considered as a major release mechanism. One critical question regarding the presence of ATP in vesicles is: how does the ATP become loaded and concentrated in these vesicles to high millimolar concentrations? We hypothesize that ATP channels and transporters may function to transport or load ATP into these vesicles and concentrate the ATP within. As an anion, ATP transport into the vesicle could be facilitated by  $H^+$ -ATPases, which would transport and concentrate  $H^+$  in

the vesicle lumen. ATP would follow to preserve macroscopic electroneutrality across the vesicle membrane. It is important to note that, once a vesicle fuses and release its quanta of ATP, that ATP channels and/or transporters would then be inserted and present in the plasma membrane to drive ATP release further. These are the same channels and transporters that served to load the vesicle before its fusion. The potential issues surrounding the loading, fusion, and release of ATP from secretory vesicles is shown in Fig. 2.

## 3. Innovative methods to detect autocrine ATP release and signaling

### 3.1. Indirect pharmacological screening for extracellular nucleotide and nucleoside release and signaling: nucleotide and nucleoside scavengers

A potent and effective way of blocking autocrine and paracrine signaling by nucleotides and nucleosides is with inclusion of scavengers for purinergic agonists. Hexokinase (with inclusion of 5 mM glucose) will scavenge ATP in preparations, yielding ADP and donating the terminal or gamma phosphate of ATP to glucose forming glucose-6-phosphate. This maneuver eliminates ATP from solutions and prevents any endogenous ATP released in cell or tissue preparations from interacting with P2 receptors that prefer ATP. ADP is a better agonist at some P2Y receptors and a weak agonist for some P2X receptors. As such, in lieu of hexokinase, apyrase, an ATPase/ADPase, would eliminate ATP and ADP from solutions rapidly. However, apyrase generates 5'-AMP, a precursor of adenosine activating P1 adenosine receptors. Thus, a final maneuver is the inclusion

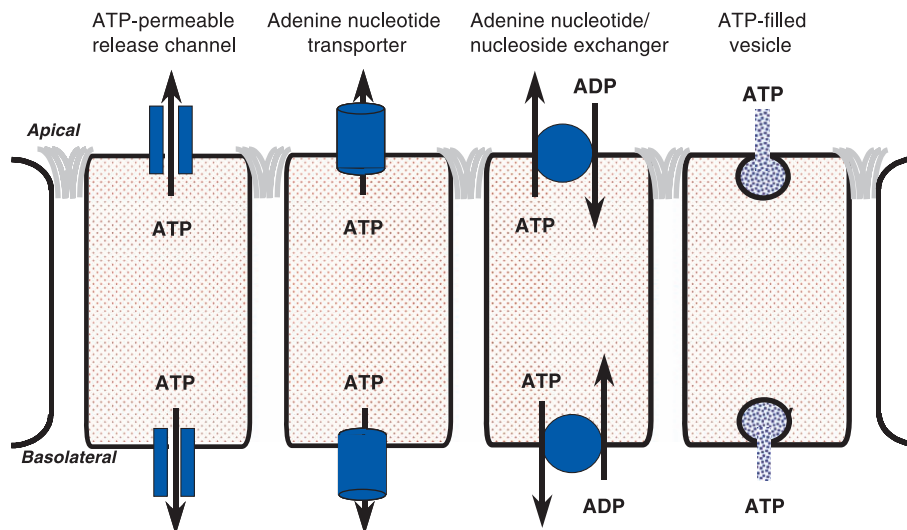


Fig. 2. Possible ATP release mechanisms in apical and basolateral membranes of polarized epithelia. From left to right: ATP-permeable release channels may be present in each membrane domain that are likely anion-permeable channels. Plasma membrane forms of the mitochondrial porins or VDACS may be candidates. Adenine nucleotide transporters may also exist that may be carriers or permeases not unlike the ABC transporters, CFTR, mdr, or MOAT. Adenine nucleotide/nucleoside exchangers are located in the mitochondrial membrane and may also be expressed in the plasma membrane. ATP-filled vesicles may also be present in epithelia as they are in neurons, neuroendocrine cells, mast cells, and platelets.



of adenosine deaminase, which converts active adenosine into inert inosine.

### 3.2. Indirect pharmacological screening for extracellular nucleotide and nucleoside release and signaling: global P2 and P1 receptor antagonists

An alternative maneuver to prevent autocrine or paracrine activation of purinergic receptors are non-selective or global antagonists that block most if not all P2 or P1 receptors. The best approach for broad blockade of P2 receptors is suramin. It is noteworthy, however, that suramin poorly inhibits certain P2X receptor subtypes, is also a weak antagonist for growth factors, and may also block other classes of calcium entry channels besides P2X receptor channels. In this case, pyridoxal phosphate-6-azophenyl 2',4'-disulfonic acid (PPADS) can be used together with suramin in a cocktail. Nonetheless, both PPADS and suramin are ecto-nucleotidase inhibitors complicating their use in indirect assays [43]. In addition, PPADS and trinitrophenyl-ATP (TNP-ATP), another P2X-selective agonist, change the color of the Ringer's solution and cannot be used in fluorescence experiments.

A similar approach can be taken for adenosine receptors. CPX (also known as DPCPX or 8-cyclopentyl-1,3-dipropyl-xanthine) is a broad specificity antagonist of P1 receptors.

## 4. Novel and direct assays to detect ATP release from cells

Ultimately, direct measurement of extracellular ATP in solution is the preferred course of action to study whether a cell or tissue is releasing ATP in a physiological or biological manner. There has been a recent revolution in the establishment of assays that can detect extracellular ATP signaling in cells and tissues. This has involved either adaptation of the luciferase/luciferin bioluminescence detection assay from the test tube to “sense” ATP release in biological preparations, the linkage of luciferase to cells via protein A and epitope antibodies to “sense” ATP release in the cell surface microenvironment, the development of the PC-12 pheochromocytoma cell as a “biosensor” that can measure ATP, and the invention of an atomic force microscopy (AFM) probe coated with myosin or luciferase to “sense” ATP.

### 4.1. Assays to detect ATP release: luciferase/luciferin bioluminescence detection

Firefly and *Renilla* luciferase and luciferin have been used for decades to measure ATP in preparations. Laboratories have taken aliquots of extracellular solution bathing cells and tissues, mixed these samples with luciferase and luciferin, and measured bioluminescence in a multi-step process. This has yielded much interesting and novel information. However, many laboratories have gone a step

further and included the luciferase/luciferin detection reagent directly into the medium or solution bathing the cells, cell monolayer, or tissue and measured ATP release.

Schwiebert et al. developed such an assay for detecting or “sensing” ATP release in real-time from non-polarized cells and more importantly, for polarized epithelial and endothelial cell monolayers grown on filter supports [17,19,21,44,45]. The same real-time assay has been used extensively by Fitz et al. to study ATP release from hepatocytes and cholangiocytes [20,35,46,47]. Both laboratories have demonstrated that cells release ATP constitutively under basal or unstimulated conditions and elevation of cyclic AMP (cAMP), increases in cytosolic calcium, and hypotonic challenge trigger further ATP release, while hypertonicity inhibits ATP release. Via this assay, it was shown that ATP release and signaling is lost in the apical microenvironment of CF monolayers versus controls [17,19], while ATP release and signaling in polycystic kidney disease (PKD) monolayers was as great or greater in the apical microenvironment and greater in the basolateral environment [21,44].

A groundbreaking study has applied luciferase/luciferin bioluminescence detection technology to a tissue preparation. Sorensen and Novak [48] used confocal imaging of the bioluminescence generated from ATP being catalyzed by luciferase. They studied individually dissected pancreatic acini to measure ATP release and signaling in this “in vivo-like” preparation. They showed elegantly that mechanical stimuli, hypotonic challenge, and carbachol, an agonist that increases cytosolic calcium in this tissue, all increased ATP release. They were also able to show intracellular ATP store depletion through caged ATP as well as quinacrine, which labeled a similar ATP pool. It was estimated that ATP release upon cholinergic stimulation generated an ATP concentration of 9  $\mu$ M in the acinus. It was postulated that this acinus-derived ATP not only could modulate acinar cell function in an autocrine manner but could also diffuse to the pancreatic ducts in acinar secretions to stimulate P2 receptors in a paracrine fashion. This laboratory, as well as our own, are trying to take this technology to more in vivo-like tissue preparations to relate the in vitro findings in cultured cell monolayers to freshly dissected tissues. One important issue is cell damage during the dissection. Novak et al. showed elegantly via multiple lines of evidence that this possibility was eliminated carefully in their work.

A limitation of the above methods of extracellular ATP “sensing” or detection is the fact that the luciferase/luciferin detection reagent is solubilized in the solution or medium; however, because of unstirred layers both in the cell cultures and cell monolayers in vitro and the freshly dissected tissues, the assay may miss ATP released into the microenvironment immediately above the cell surface. Beigi et al. [49] were aware of this issue and the fact that the ATP might be degraded by ecto-ATPases (ecto-apyrases) on the cell surface before it could be detected in bioluminescence assays. As such, they used molecular biology to develop a

conjugate that linked luciferase to protein A. This conjugate, through protein A chemistry, could be linked to any antibody that could be bound to an extracellular epitope of any cell surface antigen, usually a transmembrane protein. They took advantage of the fact that many extracellular epitope antibodies have been created for cell surface antigens on hematopoietic cells. They showed that they could detect micromolar amounts of ATP being released under basal conditions and that inhibition of ecto-ATPases could prolong the bioluminescence signal. Theoretically, this luciferase/protein A conjugate could be targeted to any cell surface antigen that had a well-characterized and high-affinity antibody that recognized an extracellular epitope. Targets may include CFTR or other ABC transporters implicated in the facilitation of ATP release, CD39 and other ecto-ATPases where the competition between degradation and detection could be studied, or P2 receptors themselves to sense what the local ATP concentration is at the receptor when it is engaged and activated.

#### 4.2. Assays to detect ATP release: “biosensors”

Hazama et al. used much ingenuity in developing a technique where the PC-12 cell, a pheochromocytoma cell line was exploited to be used as a “biosensor” because it expressed the P2X2 receptor and, possibly, other P2 receptors as well [50]. The initial paradigm developed involved establishing a whole cell patch-clamp recording designed to

record to P2X receptor channel currents. Then, the PC-12 cells was detached from the substrate (PC-12 cells do not attach well to tissue culture plastic as it is), and a single PC-12 cell, still in whole cell configuration, was moved into close proximity with a cultured cell of interest. If P2X receptor channels opened and current was recorded, then this would suggest that the cultured cell was releasing ATP. Hazama et al. [50] did this originally with pancreatic  $\beta$  cells to show that micromolar ATP was released along with insulin in response to glucose. Hazama et al. [51] have also applied this assay to non-CF versus CF cells as well as intestinal 407 cells. They have shown that ATP release is impaired in CF cells or in cells lacking CFTR when compared to controls.

Liu et al. [30] have extended this assay to study macula densa signaling in an *in vivo*-like preparation. Peti-Peterdi in Bell's group dissects nephron preparation in which the cTAL with the small patch of macula densa cells is attached to the glomerulus of the same nephron. Using the PC-12 “biosensor” cell either in whole cell patch-clamp mode or loaded with Fura-2 to measure calcium influx through P2X receptor channels, this group has shown that ATP is released from the serosal or basolateral aspect of the macula densa cells. Patch-clamp evidence suggests the presence of a maxi anion channel with significant permeability to ATP as the principal ATP release mechanism in macula densa. As with the AFM method below, the PC-12 cell “biosensor” method provides a probe that could be used in many other cultured or freshly dissected tissue culture preparations.

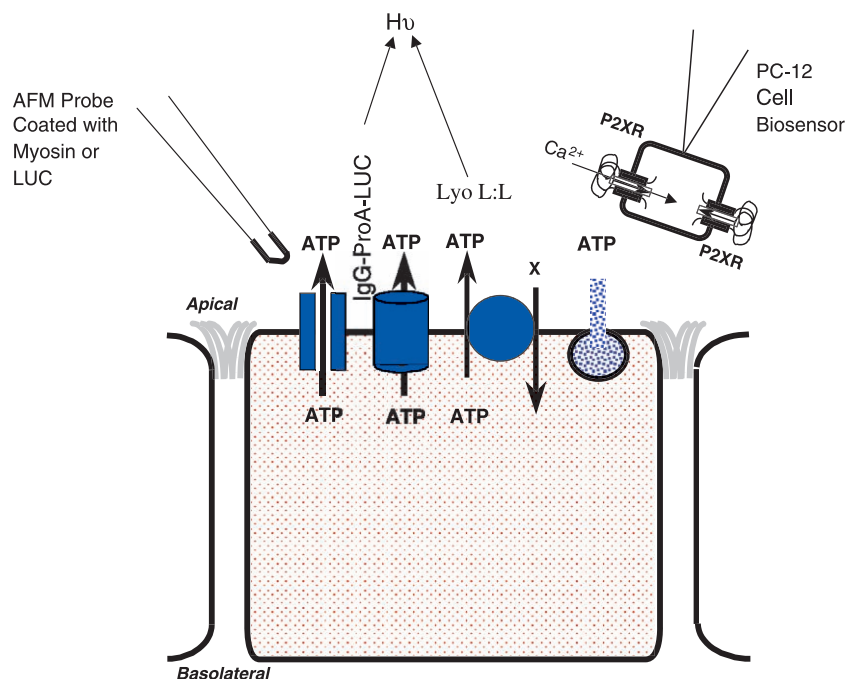


Fig. 3. Schematic summary of current real-time detection methods for released ATP. From left to right: AFM probe coated with a myosin fragment or luciferase where a conformational change and/or heat generated from the reaction (catalyzed by ATP) causes tip vibration. Luciferase linked to protein A and conjugated to an extracellular epitope IgG antibody at the cell surface or lyophilized luciferase/luciferin reagent resuspended in the medium bathing a monolayer are also used to detect released ATP in real-time within a luminometer or by microscopic imaging. A PC-12 cell loaded with Fura-2/AM or held in the whole cell patch-clamp configuration also can detect released ATP through activation of its endogenous, extracellular ATP-gated P2X2 receptor.

Recently, a different functional biosensor was developed by Schneider et al. [52] for real-time ATP measurements, which allows for the direct detection of ATP on the surface of living cells. This sensor incorporates ultra high surface topography measurements using the principles of AFM and, at the same time, allows one to scan for changes in ATP concentrations only angstroms from the surface of cells. With the implementation of this new technology, one can obtain accurate and reproducible recordings from living cells of both the topography and surface ATP concentrations in real-time. By developing the concept of a biosensor attached to the surface of an AFM cantilever, it is now possible to generate specific biosensors that can probe the surface environment of living cells under resting and experimental conditions and record changes in both the topography and the microenvironment surrounding the cell membrane.

Using this assay, Schneider et al. [52] were able to demonstrate that CF airway cells stably complemented with wild-type CFTR released ATP, measured as localized changes in ATP concentration. These changes in ATP appeared as lines across the surface of the cells that were actively secreting ATP. This aspect was intriguing to this group, as one might have expected that the cell would have single “hot spots” where the ATP was being released. By calculating the small volume that the tip actually occupies, we could determine that we would need around four molecules of ATP to be present to activate the tip [52]. As it is now possible to attach biologically active molecules to the tip of the AFMP, it may now be possible to develop a series of “biosensors” that can detect other reactive effluents from cells. This group is currently developing a tip that would allow incorporation of luciferase on the surface of the tip. This would allow one to detect relative changes in fluorescence of the tip as well the deflection of the probe due to the heat generated by the luciferase reaction. One could then directly calculate the level of ATP being released per unit time (J.P. Geibel, personal communication).

Fig. 3 summarizes the known approaches to detection of released ATP in cell and tissue preparations. In our view, multiple approaches are prudent to validating that autocrine and paracrine nucleotide signaling is occurring in a preparation. Often, this includes pharmacological blockade of purinergic signaling along with luciferase-based detection of the released nucleotide.

## 5. Heptahelical nucleotide-activated G protein-coupled receptors (P2YRs)

Early work, primarily in the cardiovascular system, recognized first the potent extracellular actions of ATP and adenosine [2]. Burnstock [4,53] later identified and proposed the concept of “purinergic” nerves and “purinergic” receptors. The early classification proposed two major groups, P2 receptors (for ATP as the agonist) and P1 receptors (for adenosine as the agonist) [54]. Subsequently,

rank order potency profiles with different nucleotides and nucleotide analogs (as well as nucleoside and nucleoside analogs) revealed distinct pharmacological subtypes for the P2 and P1 subgroups. For example, P2U (later renamed P2Y<sub>2</sub>) was characterized by UTP as its best agonist. Permeabilization of cells by ATP and other nucleotide agonists was classified as being mediated by the P2Z and/or P2X receptors. P2T was a receptor classified early on in thrombosis by platelets. It has still not been identified at the molecular biological level. P1 receptors were quickly split into two groups: A1 (for adenosine receptors that couple via G proteins to phospholipases) and A2 (for adenosine receptors that couple via G proteins to adenylyl cyclases).

The P2Y family of heptahelical or serpentine receptors is well characterized and has been reviewed extensively. The reader is referred to many seminal reviews on P2YRs for more detailed information [55–63]. Extracellular ATP-gated P2X receptor channels will be dealt with in detail below.

Rank order potency of agonists has always been the first method of differentiating P2YR subtypes. It was and still is an imperfect science. However, in the absence of a large array of selective agonists and a total absence of selective antagonists, it was all purinergic receptor laboratories had in their arsenal. Molecular biology verified this imperfect science, when the P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors were cloned. In the old nomenclature, the P2YR (later renamed P2Y<sub>1</sub>) was classified as being activated equally well by ATP and ATP analogs as well as ADP and ADP analogs. The analogs that were most effective were 2-methylthio ATP (2-MeS-ATP) and 2-MeS-ADP as well as ATPγS and ADPβS. Very recently, two new antagonists have been developed that are selective for P2Y<sub>1</sub>, MRS 2179 and MRS 2269 [55]. P2Y<sub>1</sub> (together with P2Y<sub>12</sub>) remains a candidate for being the actual P2T receptor on platelets, because ADP is the major co-agonist with thrombin that promotes platelet self-aggregation. The P2Y<sub>2</sub> receptor (P2UR in the old nomenclature) is identified by its equal or greater affinity for UTP and UTPγS versus ATP, while ADP, UDP and diphosphate analogs are ineffective. This became a less perfect diagnostic tool when the P2Y<sub>4</sub> receptor was cloned and characterized. P2Y<sub>4</sub> also binds UTP, UTPγS, and ATP. In contrast, P2Y<sub>6</sub> binds UDP with higher affinity than any other agonist. P2Y<sub>3</sub> is a species homolog of P2Y<sub>6</sub>. More recently, P2Y<sub>11</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> were identified by homology cloning and were verified to be nucleotide receptors [64]. Interestingly, the UDP-glucose receptor that was originally cloned from human myeloid cells is structurally related to the P2Y receptors. Thus, this receptor has been tentatively included in the P2Y receptor family as P2Y<sub>14</sub> [65]. However, the pharmacology is quite broad again for these receptors and exhibits a similar profile to the P2Y<sub>1</sub> receptor. Verification of nucleotide receptors is an important point, because cDNA clones with significant homology to known G protein-coupled nucleotide receptors have subsequently been characterized and found not to be nucleotide receptors. These include P2Y<sub>5</sub>, P2Y<sub>7</sub> (found to encode the leukotriene B4

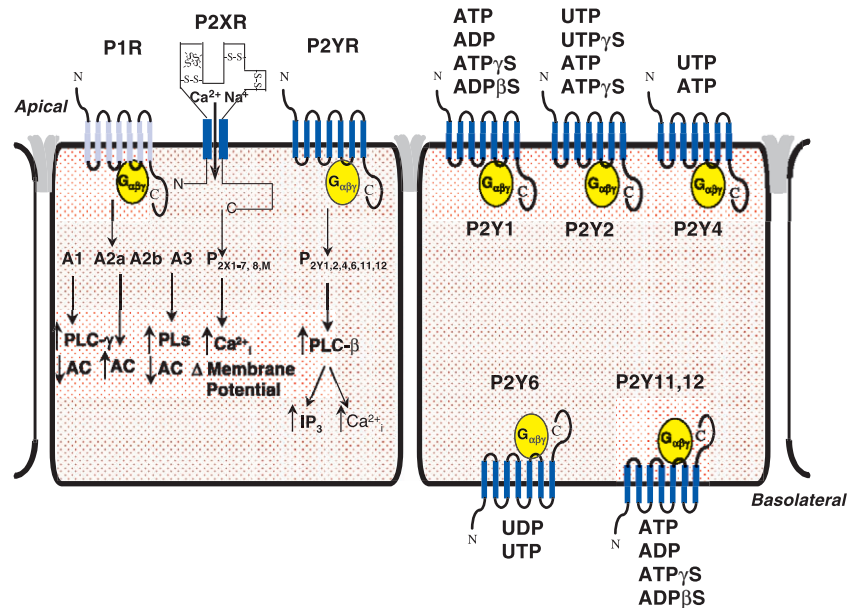


Fig. 4. Illustration of the P2Y receptor subtype classification. The imperfect science of using nucleotides or nucleotide analogs to decipher the presence of different P2Y subtypes is shown in this cartoon. Only UDP is really truly selective for P2Y<sub>6</sub>. P2YRs can be dissected better from P2XRs and P1Rs by virtue of their signal transduction cascades (PLC=phospholipase C; AC=adenylyl cyclase) and the nature of the receptor-mediated increase in cytosolic calcium.

receptor), P2Y<sub>8</sub>, P2Y<sub>9</sub>, and P2Y<sub>10</sub>. This may relate to the fact that there are hundreds of known heptahelical receptors in the G protein-coupled receptor superfamily, some of which are still “orphan” and searching for an agonist. Nevertheless, in our laboratory, we probe for P2YRs by using UTP (for P2Y<sub>2</sub> and P2Y<sub>4</sub>), UDP (for P2Y<sub>6</sub>), and ADP (for P2Y<sub>1</sub>, P2Y<sub>11</sub>, and P2Y<sub>12</sub>).

Despite the wide-ranging and non-specific pharmacology, P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2Y<sub>11</sub> share a common signal transduction pathway involving heterotrimeric G<sub>q</sub> protein, phospholipase C $\beta$ , endoplasmic reticulum-dependent Ca<sup>2+</sup> mobilization and activation of protein kinase C. In contrast, P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub> are G<sub>i</sub>-coupled receptors and do not trigger increase in cytosolic Ca<sup>2+</sup> concentration but do activate a host of G<sub>i</sub>-initiated signaling cascades. It is interesting to note that P2Y<sub>11</sub> receptors can directly activate adenylyl cyclase and cAMP accumulation [66]. Fig. 4 illustrates the subclassification of P2Y G protein-coupled receptors. P1 adenosine receptors are also shown to be thorough. A brief summary of their classification and properties is included in this figure as well. For details concerning adenosine receptors, the reader is referred to recent reviews [60,67,68].

## 6. Extracellular ATP-activated ion channels (P2XRs)

### 6.1. Background

The fact has been known for decades that application of ATP as an extracellular agonist on a cell or tissue led to “permeabilization” of the cell plasma membrane. There

were many interpretations of this phenomenon, although a role in exacerbation of cell injury was a common conclusion. The receptors thought to underlie this effect were classified as the P2Z receptors in the early nomenclature and were thought to be a subtype of the P2Y family. P2X receptors were first distinguished from P2Y receptors based on pharmacological criteria by Burnstock and Kennedy in 1985 [69]. This early subdivision of P2 receptors was later validated based on molecular cloning and second messenger system of P2X and P2Y families [70].

In seminal papers and reviews about this original electrophysiological research on native cells, Bean et al. [71–74] showed that single channel conductances of as little as 1 picoSiemen (pS) to as large as 100 pS could be observed upon addition of ATP agonists. The different channels also had different biophysical current signatures, with differing kinetics, dependence on voltage, and rate of inactivation (later determined to be desensitization). The permeability of the channels to monovalent and divalent ions also varied. This was exceedingly frustrating, because the variability in the recordings often made this research difficult to decipher. Moreover, this issue of ATP permeabilization of cells always loomed in the background as a troubling issue. In hindsight, a subset of these channels were indeed P2XRs of varying types and, possibly, different multimeric mixtures. Other channels recorded immediately after ATP stimulation may have been separate ion channels that P2XRs and, possibly, P2Y G protein-coupled receptors, were stimulating via rapid signal transduction mechanisms.

There were also physiological reasons for believing that extracellular ATP-gated ion channels existed. Burnstock [4] proposed the existence of so-called “purinergic nerves” in



the gut and in the nervous system that were neither cholinergic nor adrenergic. By inference, the ATP that was released from the synapse had to bind to a receptor channel to propagate the action potential. Moreover, the ATP that was applied to whole cell patch-clamp and voltage-clamp preparations activated ion channels as rapidly as activation observed in the acetylcholine receptor channel in neurons and muscle. There was also a relative potency of different nucleotide agonists in the rapid activation of these ion channels, suggesting that this was a receptor-mediated effect that had physiological meaning.

## 6.2. Molecular identity

This physiological work has now been validated by molecular biology. At least nine P2X receptor channel subtypes (P2XRs, as we will refer to them) have now been cloned, identified, and characterized [75–83]. Other tissue-specific [84] and developmentally regulated P2XRs [85,86] are still emerging, as are P2XRs from lower model organisms such as zebrafish [87–89]. Transgenic knockout of P2XR genes has begun in an effort to understand their physiological roles more thoroughly. Mice lacking P2X<sub>1</sub> receptor channels expressed one major phenotype, reduced fertility due to lack of ejection of sperm from the testes into the vas deferens [90]. The interpretation was that P2X<sub>1</sub> receptors are the main purinergic receptor on testes and vas deferens vascular smooth muscle cells. The sympathetic-driven contractile response is mediated in large part by P2XRs; however, this study showed that P2X<sub>1</sub> was the principal postsynaptic receptor channel involved. Fertility was reduced by 90% in homozygous knockout mice, but not in wild-type or heterozygous mice, not because of defects in spermatogenesis, but in ejection of sperm through the vas deferens into the ejaculate [90]. Any other phenotypes in the mice were subtle and not detected as yet in this study.

Vlaskovska et al. [91] have generated a P2X<sub>3</sub>-knockout mouse. With this mouse, this laboratory has shown that two different populations of sensory neurons are impaired in their function due to a lack of P2X<sub>3</sub> [91,92]. The suburothelial plexus of the mouse bladder showed abundant staining for P2X<sub>3</sub>. Although ATP was released similarly in response to bladder distension in P2X<sub>3</sub> *+/+* versus P2X<sub>3</sub> *-/-* mice, the bladder afferent nerve activity was attenuated in response to distension [77]. The loss of this activity in the P2X<sub>3</sub> *-/-* mice could be rescued by exogenous ATP analogs or ATP itself, while normal activity in the P2X<sub>3</sub> *+/+* afferents was blocked by TNP-ATP, PPADS, and capsaicin [91]. These data suggest that P2X<sub>3</sub> is a major neural sensory receptor on a population of bladder nerve fibers that transduce bladder distension into an electrical signal. Specialized neurons of the dorsal root ganglia respond to ATP as a principal neurotransmitter by a mechanism that is transduced by different P2XR subtypes, P2X<sub>2</sub>, P2X<sub>2</sub>/P2X<sub>3</sub> heteromultimers, and P2X<sub>3</sub>. Zhong et al. [92] also examined the activity of DRG and nodose neurons

(specialized neurons of the DRG involved in pain perception) in P2X<sub>3</sub> *-/-* mice. Because P2X<sub>3</sub> has a distinctive, rapidly desensitizing response to ATP, patch-clamp electrophysiology of the neurons was employed to look for differences between P2X<sub>3</sub> *+/+* and P2X<sub>3</sub> *-/-* cells. All P2X<sub>3</sub> *-/-* DRG neurons failed to desensitize after ATP stimulation, and both subsets of neurons failed to respond to  $\alpha\beta$ -methylene ATP. The responses that were persistent in the P2X<sub>3</sub> *-/-* neurons were consistent with intact, endogenous expression of P2X<sub>2</sub>. These studies showed that, although DRG and nodose neurons remained responsive to ATP, the nature of the neuronal sensory response to the ATP neurotransmitter in the spinal cord is significantly altered and more long-lived.

## 6.3. A member of the two transmembrane-spanning cation channel superfamily

P2XRs were assumed to be a subtype of the heptahelical P2Y family. Instead, P2XRs were predicted to form two transmembrane-spanning ion channels with intracellular N- and C-termini. Once more, two-thirds of the molecular mass of this protein was predicted to be extracellular. This overall structure made P2XRs closely related to the ENaC/degenerins family of cation channels found in mammalian cells and in the nematode, *C. elegans* [93–95]. Fig. 5 illustrates this similarity. In the extracellular domain of each cation channel family, 10 conserved cysteines are found in P2XRs, while 14 conserved cysteines are found in the ENaCs. Each extracellular domain is highly glycosylated, with at least three putative glycosylation sites [93–96]. This large extracellular domain, which accounts for two-thirds of the molecular mass of P2XRs, is shared by P2XRs and ENaCs; however, this domain is divergent from the large family of inwardly rectifying K<sup>+</sup> (IRK) channels. P2X<sub>2</sub> and  $\alpha$ ENaC appear to share the PPXXY motif that is mutated in Liddle's disease in  $\alpha$ ENaC and is a recognition site for ubiquitination [93,95]. What is still unclear in both P2XR and ENaC families is the amino acids critical to the cation channel pore. Extracellular to the second transmembrane domain of ENaC is a putative H2 domain that has a GGQLG sequence that is somewhat similar to the GYG motif found in the P loop (equivalent to H2 domain) of two transmembrane-spanning IRK channels. P2XRs are not thought to have a P loop; however, recent alignment of the sequences of P2XRs did reveal a GVG motif in the second transmembrane  $\alpha$ -helix of all P2XRs except P2X<sub>7</sub> [97]. It is important to note that there is little or no overall nucleotide sequence homology between any P2XR subtype and any ENaC subtype, despite the very similar structural characteristics.

The similarities in topology of P2XRs and ENaCs also extend to their cell physiology. As members of the two transmembrane-spanning ion channel superfamily (see above), P2XRs were long suspected to form homomultimers or, in some cell models, heteromultimers of mixed P2XR subtypes. ENaCs form fully functional amiloride-sensitive

- Display Eerily Similar Topology
- Form Homo- and Heteromultimers
- Have Two Cysteine-rich Regions in their Extracellular Domain  
( $\alpha\beta\gamma$ ENaCs Have 14 Conserved Cysteine Residues in this Domain)  
(P2XRs Have 10 Conserved Cysteine Residues in this Domain)
- Are Heavily Glycosylated on Multiple N-linked Glycosylation Sites
- Two-Thirds of the Molecular Mass on ENaCs and P2XRs Is in the Extracellular Domain
- $\alpha$ ENaC and P2X2 Have a “PPPLY” Motif in Their C-Termini

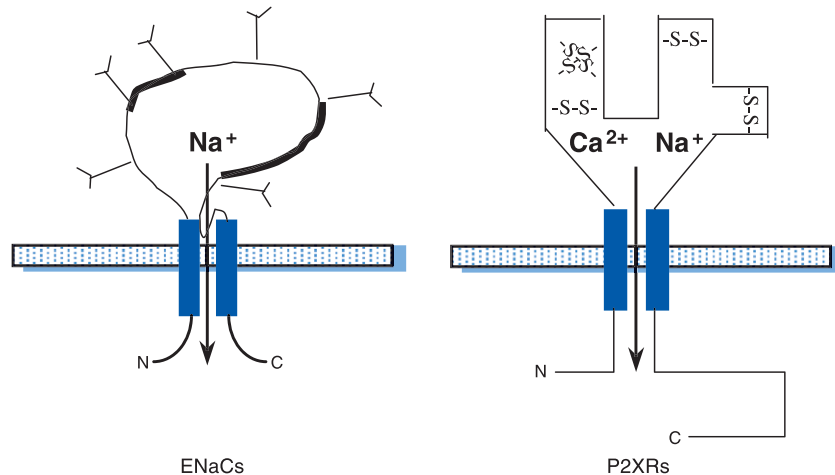


Fig. 5. Close and detailed comparison between P2XRs and ENaCs. These two transmembrane-spanning cation channel subfamilies exhibit similarity in their topology and, possibly, their overall structure. Other biochemical and cell biological similarities are listed, keeping in mind that there is little or no overall DNA or amino acid sequence homology. One major difference is the fact that the cation permeability/selectivity of each subfamily is very different.

and highly  $\text{Na}^+$ -selective channels when configured as an  $\alpha\beta\gamma$ -ENaC heteromultimer [93,95]. Torres et al. [98–101] explored multimerization elegantly with parallel biochemical and physiological methods in the HEK-293 cell heterologous cell system with multiple studies. The most elegant of these studies was the examination of the ability of all P2XR subtypes to oligomerize or multimerize [99]. Co-immunoprecipitation of epitope-tagged P2XRs was undertaken to probe the ability of P2XRs to form homomultimers and heteromultimers. HA and FLAG biochemical tags added to the C-terminus of each P2XR subtype did not affect cation channel function or other properties such as desensitization. The exception in this work was P2X<sub>6</sub>, where even the wild-type construct failed to generate cation currents. Again with the exception of P2X<sub>6</sub>, all other P2XRs tested (P2X<sub>1</sub> through P2X<sub>5</sub> and P2X<sub>7</sub>) were capable of forming homomultimers. The authors maintain that the inability of P2X<sub>6</sub> to form oligomers may explain why it also fails to form functional channels. The stoichiometry of these multimers is unknown; however, dimers, trimers, and tetramers have been postulated [99]. With regard to heteromultimers, all P2XRs were capable of forming multimeric complexes with other subunits, with the notable exception of P2X<sub>7</sub>. A helpful table in this paper [99] summarizes which P2XR subtypes oligomerize with specific other P2XR subtypes. P2X<sub>1</sub> and P2X<sub>2</sub> co-assemble with themselves as homomultimers and with P2X<sub>3</sub>, P2X<sub>5</sub>, and P2X<sub>6</sub>,

but not P2X<sub>4</sub> or P2X<sub>7</sub>. P2X<sub>3</sub> co-assembles with P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>5</sub>, but not with P2X<sub>4</sub>, P2X<sub>6</sub>, or P2X<sub>7</sub>. P2X<sub>4</sub> expressed at very high levels in the HEK-293 cell system, making it difficult to assess in this analysis relative to the other subtypes. However, when the conditions were made favorable, P2X<sub>4</sub> was found to oligomerize only with itself and with P2X<sub>5</sub> and P2X<sub>6</sub>. This result was very helpful to our laboratory, which found that P2X<sub>4</sub> and P2X<sub>5</sub> were most abundantly expressed in human vascular endothelial cells and in human and rodent airway, gastrointestinal, and kidney epithelial cell models. There is some preliminary evidence emerging from other laboratories that P2X<sub>6</sub> may also be a prominent epithelial subtype. Unfortunately, the lack of an antibody and the lack of an ability for our degenerate RT-PCR to detect P2X<sub>6</sub> has not allowed us to assess its expression. P2X<sub>5</sub> co-assembled with every other P2XR subtype, except P2X<sub>7</sub>. P2X<sub>6</sub> co-assembles with P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>4</sub>, but fails to interact with P2X<sub>3</sub>, itself, or P2X<sub>7</sub>. These seminal results solidify a plethora of the functional studies [98,99,101–106] that show physiological evidence for heteromultimeric assembly of unique pairs of P2XR subunits.

#### 6.4. P2XR biophysics

There is a profound difference between P2XRs and ENaCs with regard to selectivity of cation permeation.

The  $\alpha\beta\gamma$ -ENaC heteromultimer is the most highly  $\text{Na}^+$ -selective cation channel ever described. In sharp contrast, P2XRs are non-selective cation channels for monovalent and divalent cations. P2XRs are calcium-permeable non-selective cation channels that, like the glutamate, NMDA, and kainate receptor channels at excitatory synapses, are gated by an extracellular agonist, show divalent and monovalent permeation, and divalent cation block. P2XRs are thought to trigger signaling by mediating calcium influx from extracellular stores and by changing the plasma membrane potential; however, other signaling mechanisms may be governed by ATP-gated receptor channels. P2XRs, as well as the P2Y G protein-coupled receptors, also stimulate other ion channels in native cells, complicating their definition in specific cell models.

Ding and Sachs [107] studied elegantly the biophysics of the P2X<sub>2</sub> channel protein, expressed in HEK-293 cells devoid of endogenous P2XRs when maintained as isolated cells. Outside-out patches were performed so that the ATP agonist had free access to the extracellular domain of the receptor channel protein. While monovalent cations were permeant in a sequence that was  $\text{K}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{Na}^+ > \text{Li}^+$ , divalent cations also entered the pore. However, divalent cations such as  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Ba}^{2+}$  also blocked the channel in a manner characterized as “fast block,” reducing the amplitude of the single channel currents. Importantly, organic cations such as NMDG<sup>+</sup>, Tris<sup>+</sup>, TMA<sup>+</sup>, and TEA<sup>+</sup> were virtually impermeant. The channels were “flickery,” in that they opened in brief bursts. The single channel conductance was 49 pS in the presence of  $\text{K}^+$  and 35 pS in the presence of  $\text{Na}^+$ . P2XR  $\text{Na}^+$  currents were partially blocked by  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ . A subsequent paper showed that divalent cations also speeded inactivation of the P2X<sub>2</sub> channels [108].

### 6.5. Key amino acid residues within P2XRs

Site-directed mutagenesis has been used to probe many regions critical to the activity of P2XR channels. With regard to cation permeation through the pore, 5 amino acid residues in a 20-amino acid stretch corresponding to the first transmembrane  $\alpha$ -helix (H33, R34, I50, K53, and S54) were implicated in cation binding and permeation within the P2X<sub>2</sub> receptor channel [109,110]. Another study of P2X<sub>2</sub> channels in HEK-293 cells probed a larger stretch of amino acids flanking and within the first transmembrane segment [111]. Their cysteine-scanning mutagenesis revealed that D15, P19, V23, V24, G30, Q37, and F44 were involved in cation binding and permeation. Migita et al. [112] also probed the second  $\alpha$ -helical transmembrane domain for residues key in divalent cation binding and conduction through P2XRs.  $\text{Ca}^{2+}$  permeability through P2X<sub>2</sub> expressed in HEK-293 cells was thought to be mediated by amino acids with negatively charged side chains, D315 and D349. This had no effect on divalent cation permeation. Rather, the size of side chains that were exposed to the pore of the

channel was critical. Mutation of polar threonines at position 336 and 339 as well as a serine 340 affected cation permeation profoundly. The largest changes were induced by replacement of these residues with a tyrosine, whose bulky side chain prevented  $\text{Ca}^{2+}$  movement altogether. Taken together, these three studies by Egan and Voigt and one study by North et al. showed that the two transmembrane  $\alpha$ -helices cooperate in conduction of monovalent and divalent cations through the pore.

In addition to the evidence generated by Haines et al. [109] regarding ATP gating of the channel by binding to the extracellular face of the first transmembrane segment, Jiang et al. [113] also addressed this issue in the P2X<sub>2</sub> receptor expressed in HEK-293 cells. Alanine-substitution mutagenesis on 30 polar residues in the presumptive extracellular domain revealed two positively charged lysine residues (L69 and L71) near the same region proximal to transmembrane domain 1 implicated by Egan and Voigt. Substitution with cysteines for these lysines and other flanking amino acid residues showed similar effects. These experiments also implicated S65 and I67 in ATP binding and gating of the P2X<sub>2</sub> channel. In particular, I67 was identified to be critical to the ATP-binding site [113]. Perhaps, anionic ATP is attracted to and bound by these lysines and the flanking serine and isoleucine help stabilize this interaction. Upon binding, ATP may also mask these closely coupled lysines with positively charged side chains, removing steric hindrance and allowing cations to move from the external channel vestibule into the pore. Ennion et al. [114] studied the extracellular domain of the P2X<sub>1</sub> receptor, searching for residues critical for ATP binding. This study and the one above came out concurrently. They too targeted conserved lysines and arginines, postulating that positively charged side chains might attract and/or bind anionic ATP. Many were irrelevant; however, like the P2X<sub>2</sub> receptor studies above, K68 and K70 external to transmembrane domain 1 and R292 and K309 external to transmembrane domain 2 were key to ATP recognition. Taken together, these two studies on different P2XR subtypes show that the ATP binding pocket is indeed at the extracellular face of the cation pore close to the channel vestibule.

P2X<sub>1</sub> and P2X<sub>3</sub> receptor channels desensitize rapidly upon binding ATP and opening, while the other major subtypes (P2X<sub>2</sub> and P2X<sub>4</sub> through P2X<sub>7</sub>) inactivate slowly or not at all. Koshimizu et al. [115] swapped a stretch of six amino acids of P2X<sub>3</sub> and for P2X<sub>4</sub> with the corresponding stretch of arginine 371 through proline 376. P2X<sub>4</sub> sequence substitution slowed receptor desensitization, while P2X<sub>1</sub> and P2X<sub>3</sub> sequence substitution caused rapid desensitization. Co-expression of P2X<sub>2</sub> and P2X<sub>3</sub> caused a phenotype that had delayed desensitization, a phenotype intermediate to P2X<sub>2</sub> and P2X<sub>3</sub> expressed alone. Thus, this highly charged stretch of amino acids in the C-terminus plays a significant role in desensitization. Boue-Grabot et al. [116] identified a protein kinase C phosphorylation site, TX(K/R) at amino acids 18 through 20 in the N-terminus of the P2X<sub>2</sub>

receptor, as critical for regulation of receptor desensitization. Normally, a slowly or poorly desensitizing P2X subtype, mutation of the N-terminus of P2X<sub>2</sub> (T18A, T18N, or K20T) showed rapid desensitization. Phosphorylation of this site within the N-terminus was verified biochemically. Examination of the N-terminal sequences of all P2X subtypes reveals that P2X<sub>1</sub> and P2X<sub>3</sub>, rapidly desensitizing subtypes, lack this threonine at position 18, while the other more slowly desensitizing subtypes possess this conserved PKC site. Still another laboratory generated data suggesting that the hydrophobic transmembrane segments were critical in desensitization [117]. Chimeric swapping of the TM domains from P2X<sub>1</sub> and P2X<sub>3</sub> with P2X<sub>2</sub> affected desensitization markedly. Taken together, these results suggest that all regions of the P2X receptor accessible to the cytosol are involved in receptor desensitization following ATP binding and channel opening.

#### 6.6. *Wide and robust expression of P2XRs*

P2XR subtypes are expressed abundantly in brain and spinal cord, and the most comprehensive analysis of their relative expression in these tissues was from Collo et al. [82]. The functional role of P2XRs in different excitable cell preparations will be addressed in more detail below. It was once thought that P2XR expression was restricted to excitable cells. Although the P2XR as an excitatory postsynaptic receptor channel is easy to envision and characterize, the physiological roles of P2XRs in non-excitable cells were more difficult to visualize. Even if *in situ* hybridization revealed staining for P2XRs on non-excitable cells, this was met with some skepticism.

Emerging studies have revealed abundant expression of P2XRs on non-excitable cells and tissues. Although sensory organs do contain neurons that convey sensory inputs to higher neural centers, evidence has emerged that P2XRs are expressed in other more specialized, non-excitable cell types in inner ear, retina, and taste bud. Multiple P2XRs have been localized to spiral and vestibular ganglia of the inner ear, the cochlear nucleus, and primary auditory neurons [118–120]. In addition, P2X<sub>2</sub> receptors or splice variants of P2X<sub>2</sub> [121] have been found biochemically, molecularly, and functionally in Dieters' cells of the cochlea [122], organ of Corti within the cochlea [123], outer hair cells [119], and epithelial cells lining the stria vascularis, Reissner's membrane, and the tectorial membrane [118]. P2X<sub>2</sub> staining was also intense in the hair cell stereocilia, indicating a key role in sound transduction [124]. In the retina, P2XRs are also abundant [125–130]. In addition to transduction of pain, light, and sound, P2XRs have also been identified in circumvallate fungiform papillae of the rat tongue [131].

The role of P2X<sub>1</sub> in the smooth muscle cells of the testes and vas deferens has already been well documented with regard to cloning of P2X<sub>1</sub> and the P2X<sub>1</sub> knockout mouse. The urinary bladder appears to be another important tissue regulated by local ATP release and P2XRs. In detrusor,

ureteral, and bladder blood vessel smooth muscle, P2X<sub>1</sub> immunoreactivity was observed routinely and markedly on the smooth muscle cell membrane [132]. While P2XR expression remains and is pronounced in smooth muscle, P2XR expression in skeletal muscle is notable during early stages of development, while expression fades during the postnatal period and in adult tissue [133,134]. Recently, P2X<sub>4</sub> was found in chick cardiac muscle myocytes. A positive ionotropic effect of ATP on heart muscle may be mediated by this P2XR subtype and allow ATP-induced calcium influx that was blocked by antisense oligonucleotides to P2X<sub>4</sub> [135]. In addition, glycosylated (58 kDa) and nonglycosylated (44 kDa) forms of the transfected cP2X<sub>4</sub> receptor were found in cardiac myocyte membranes, a similar phenotype to the epithelial P2X<sub>4</sub> receptor (see below). Only the 58 kDa glycosylated form of cP2X<sub>4</sub> could be biotinylated. These results provide a new tissue where P2XR expression and function may be critical.

Very recent work has shown the presence of P2XRs in human vascular endothelial cells [45,136–140]. In the context of expression profiling in excitable tissues, hints in these initial studies led to the characterization of P2XR expression and/or function in epithelial cells *in vitro* and *in vivo* [141]. An emerging role for purinergic receptors in the cells that constitute skeletal bone is being appreciated. Bone remodeling within microenvironments is regulated by autocrine and paracrine factors and by local mechanical forces that are poorly understood. A recent and comprehensive study on P2X and P2Y receptor expression in bone cells has been performed [142]. Three studies that followed added credence to these findings [143–145].

Burnstock et al. have led the field in documenting P2X receptor expression in endocrine tissues. Initial work documented abundant P2X<sub>2</sub> receptor expression in the nuclei and neurons of the hypothalamus [146,147]. Similar work has been performed in thyroid and adrenal gland [148–151]. Petit et al. showed roles for P2Y and P2X receptors in stimulating insulin secretion from pancreatic  $\beta$  cells in the absence or presence of the requisite extracellular glucose stimulus [152]. Burnstock's group has also studied expression of all P2XR subtypes in rat testis [153]. Taken together, expression of multiple P2XR subtypes in endocrine organs argues for tight, local control of endocrine tissue function by autocrine purinergic signaling.

#### 6.7. *Biochemistry of P2XRs in native cells*

Recent studies have shed new light on P2XR biochemistry in native cells and tissue. Most of this biochemical work has been performed in heterologous cell systems with transfected and, often, epitope-tagged P2XR constructs. Recently, Hu et al. [135] made a step closer to studying native cells by studying the protein biochemistry of a transfected chick P2X<sub>4</sub> receptor in cultured embryonic ventricular myocytes. Not only did Hu et al. show compelling evidence for a role for P2X<sub>4</sub> in calcium influx from



extracellular stores and in contractile amplitude of the chick heart, they also showed the fact that P2X<sub>4</sub> receptors were resistant to various rather harsh detergents under various biochemical conditions. Initially, they verified that the P2X<sub>4</sub> receptor was found in two forms: a nonglycosylated 44 kDa form and a glycosylated 58 kDa form. Only the glycosylated form was biotinylated and reacted with streptavidin, suggesting that glycosylation was necessary for normal trafficking. Interestingly, the glycosylated form was soluble in all of the detergent combinations listed above.

Our laboratory has confirmed these results in human epithelial and endothelial cell models for the human and mouse P2X<sub>4</sub> receptor [44,45]. The novel aspect of these studies is the immunoblotting of native epithelial and endothelial P2X<sub>4</sub> receptor. Similar results have also been obtained in non-CF and CF human airway epithelial cells [154] and normal and PKD kidney epithelial cells (A.T. Boyce and E.M. Schwiebert, unpublished observations). Our laboratory has also seen higher molecular weight forms in immunoblotting that are competed away by the peptide immunogen, suggesting higher order glycosylation and/or a detergent-resistant multimer. The prominent carbohydrate addition on this large extracellular loop, elaborate disulfide bonding between conserved cysteines (see below), and the reaction with the extracellular domain of the ATP agonist as well as heavy metals such as zinc, protons, and cations (see below) suggests a complex and dynamic protein worth further investigation in native cells as well as crystallography of P2XRs.

As introduced above, the P2XR extracellular domain has two cysteine-rich regions, within which each P2XR has 10 conserved cysteine residues in identical locations. This argues for a complex, three-dimensional structure that begs to be crystallized. ENaCs have 14 conserved cysteine residues in their extracellular domains. Recent work suggests that ENaCs have Kunitz-like domains that are susceptible to protease cleavage, which is argued to be a major mode of activation for these channels [155]. Further proof for this is that protease inhibitors attenuate ENaC activity. The effect of proteases on P2XRs has not been tested.

The role of the cysteines in forming intra- and interchain disulfide bonds and creating trafficking-competent P2X<sub>1</sub> receptor channels was addressed very recently by Ennion and Evans [156]. A very important finding in this study was the inability to label wild-type receptors with MTSEA-biotin, suggesting that all 10 cysteine residues are engaged in disulfide bonds. When most single cysteines were mutated, only modest effects were observed on ATP potency at the receptor channel. However, exceptions were C261A and C270A, where the peak current amplitudes were reduced by almost 100%. This, however, was determined to be an effect on trafficking. The other single cysteine mutants did allow MTSEA-biotin labeling, suggesting that companion cysteines were not free to react with the methanethiosulfonate compound. Based on their work, they proposed the following pairs of cysteines that were disulfide-linked: C117–

C165, C126–C149, C132–C159 in the first cysteine-rich region and C217–C227 and C261–C270 in the second cysteine-rich region. Channel function is not affected significantly by these bonds; however, trafficking is severely disrupted by the elimination of the C261–C270 bond or by the C117–C165 bond together with another bond.

#### 6.8. Pharmacology and chemistry of P2XR receptor channel activity

The extracellular domain of the P2X receptor channel is a rich background for complex chemistry. As it accounts for two-thirds of the molecular mass of the receptor channel protein like its relatives in the ENaC/degenerin family, this protein can be thought of more as a receptor than a channel. In fact, its extracellular domain is so large and complex that P2XRs can also be thought of as sensors rather than receptors. Along this line of reasoning, ENaCs and degenerins may have endogenous chemical ligands that are not yet appreciated.

This chemistry begins with the binding of its gating ligand, ATP. Agonists and antagonists have been presented in the context of work discussed above. However, recent work has identified additional antagonists for P2XR subtypes, including TNP-ATP [157,158], NF-279, a suramin analog [159,160], and pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate (PPNDS) [161]. Despite these recent efforts, these antagonists mainly affect P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>3</sub> or mixtures thereof. The lack of agonists that are selective between P2Y and P2X receptor families, and the lack of agonists and antagonists that define particular P2XR subtypes (in particular, P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>6</sub>) is a major roadblock in the P2 receptor field and in the P2XR field that needs to be overcome.

Not only does the P2XR extracellular domain have sites for agonist and antagonist binding, N-linked glycosylation, cysteine bonding, and, possibly, protease cleavage, protons and heavy metals react within this extracellular domain as well. Stoop et al. [162] explored the sensitivity of four P2XR subtypes to changes in extracellular pH (pH<sub>o</sub>). P2X<sub>3</sub> homomultimers are only mildly inhibited by acidic pH (pH<sub>o</sub> of 6.3 versus 7.3) and are unaffected by an alkaline pH<sub>o</sub> of 8.3. In contrast, P2X<sub>2</sub> homomultimers and P2X<sub>2</sub>/P2X<sub>3</sub> heteromultimers show profound stimulation by acidic pH<sub>o</sub> and almost complete inhibition at alkaline pH<sub>o</sub>. The authors noted that the data pointed to a single site being modulated by protons. For P2X<sub>1</sub> and P2X<sub>4</sub>, opposite effects were observed with acidic versus alkaline pH<sub>o</sub>. Acidic pH<sub>o</sub> profoundly inhibited P2X<sub>4</sub> currents in particular, while alkaline pH<sub>o</sub> had little effect. Site-directed mutagenesis of the extracellular domain of the rat P2X<sub>2</sub> receptor revealed that acidic pH potentiated ATP stimulation more than 4-fold in wild-type channels and eight mutant channels in which extracellular histidines were mutated [163]. In P2X<sub>2</sub>, only one histidine mutant, H319A, attenuated the effect of acidic pH down to 1.4-fold. Substitution of a lysine instead of an

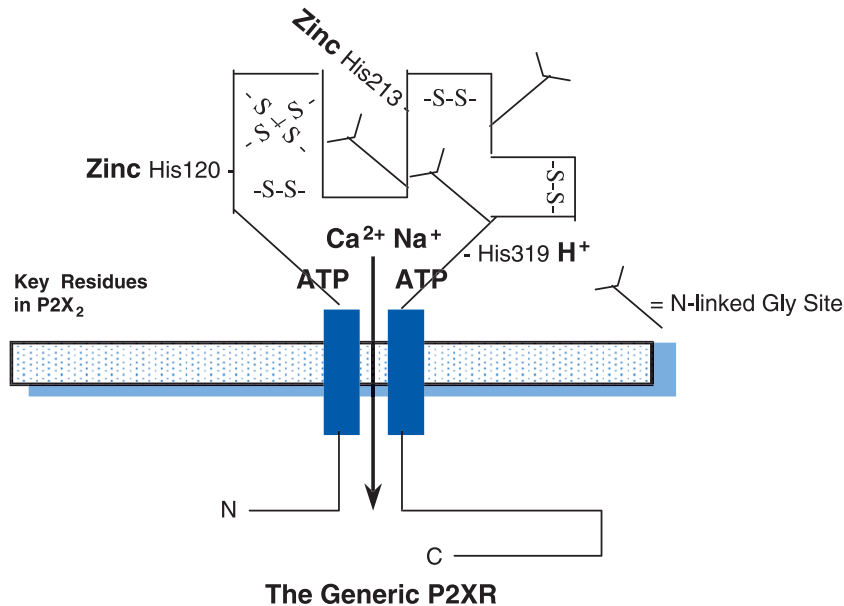


Fig. 6. Key residues within the generic P2XR. Most of the key residues highlighted in this cartoon have been defined in the P2X<sub>1</sub> and P2X<sub>2</sub> receptors. Not all characteristics may hold for the other P2XR subtypes, especially with regard to H<sup>+</sup> and Zn<sup>2+</sup> modulation. Cation binding residues were found by multiple laboratories throughout both  $\alpha$ -helical regions.

alanine reduced the EC<sub>50</sub> for ATP 40-fold. Extracellular Zn<sup>2+</sup> is known to potentiate and inhibit the responses of different P2XR subtypes to ATP. The maximal effective dose is only 20  $\mu\text{M}$ , which falls within physiological limits for this trace element. Zn<sup>2+</sup> potentiates ATP gating of P2X<sub>2</sub>, P2X<sub>3</sub>, P2X<sub>4</sub>, P2X<sub>2</sub>/P2X<sub>3</sub>, P2X<sub>2</sub>/P2X<sub>6</sub>, and P2X<sub>4</sub>/P2X<sub>6</sub>, while it inhibits ATP's effect on P2X<sub>1</sub> and P2X<sub>7</sub>. ZnCl<sub>2</sub> potentiated the response to ATP in wild-type P2X<sub>2</sub> and seven histidine mutants by 8-fold; however, in two mutants, H120A and H213A, Zn<sup>2+</sup> had no effect [163]. Because these two Zn<sup>2+</sup> reactive histidine residues are rather distant from one another within the external domain, two different sites may be required for the effect of Zn<sup>2+</sup> or the two histidines may interact with each other in a three-dimensional model of the extracellular loop. Taken together, these results suggest that multiple but different histidine residues, whose pK<sub>a</sub> falls within the range tested in these studies, bind protons and heavy metals to modulate the gating by the physiologic agonist, ATP. The opposite effects of pH and Zn<sup>2+</sup> on P2X<sub>2</sub> versus P2X<sub>4</sub> is borne out by less total histidines in P2X<sub>4</sub> and locations of these cysteines that are completely different than in P2X<sub>2</sub>. It is also possible that H<sup>+</sup> and Zn<sup>2+</sup> could act as agonists independent of ATP to open P2XRs. Fig. 6 highlights the histidine residues implicated in H<sup>+</sup> and Zn<sup>2+</sup> binding.

## 7. Physiological and pathophysiological paradigms for extracellular ATP-mediated effects in epithelia

Many of the figures included above were designed in the context of a polarized epithelium. This was no accident.

Autocrine and paracrine ATP signaling in extracellular microenvironments is robust on both the apical and basolateral sides of polarized epithelium. Epithelial cells are traditionally known as barrier cells that line the tissues of many organs of the body and separate the external environment from the interstitium. The apical membrane faces the lumen of a tissue. Often, this is the external environment, like the humidified air in the lung or a fluid-filled environment (e.g., the humor-filled eye, the ventricles of the brain filled with cerebrospinal fluid (CSF), or the endolymph-filled inner ear). The basolateral membrane faces the interstitium (e.g., basal cells, connective tissue, basement membrane, blood supply, and nerve innervation). The microenvironments on the surfaces of these two membrane domains are very different. These microenvironments often provide an ideal setting for autocrine and paracrine purinergic signaling in particular and autocrine and paracrine signaling in general. This is especially true if they are fluid-filled or fluid-covered microenvironments that have a medium to allow the autocrine or paracrine mediator to diffuse easily and rapidly.

Purinergic signaling has been implicated in the regulation of many epithelial cell functions. In addition to triggering cell signaling, mainly through cytosolic calcium and phospholipase-coupled signal transduction and, possibly, via other cellular mechanisms, extracellular nucleotide signaling also modulates the potency of other autacoids and hormones that regulate epithelial cell function. Extracellular nucleotides and nucleosides also regulate transepithelial ion transport. In general, extracellular nucleotides and nucleosides, through P2Y, P2X, and P1 receptors, stimulate secretory Cl<sup>-</sup> and H<sub>2</sub>O transport, activate K<sup>+</sup> channels,

inhibit absorptive  $\text{Na}^+$  transport, modulate acid–base transport, and potentiate regulatory volume decrease following hypotonic cell swelling. We are careful to say “in general,” because there are exceptions to this rule due, in large part, to where the epithelium resides and its function. For example, in highly secretory epithelium like choroid plexus or ciliary processes of the ciliary body of the eye, purinergic signaling may also stimulate secretory  $\text{Na}^+$  transport. Purinergic receptor-driven functions include triggering of calcium sparks and waves, potentiating ciliary beat frequency, and promoting mucus, glandular, and acinar secretion. Constitutive ATP release and signaling has been implicated in the maintenance of cell signaling “setpoints” for cytosolic calcium, phosphoinositide turnover, and arachidonic acid metabolism. Purinergic signaling may even modulate gene expression in epithelial cells via specific transcription factors.

In epithelial cells, ATP is released from the cell in a polarized manner either at the apical or basolateral cell surfaces for the purpose of autocrine and paracrine regulation of the epithelial cell monolayer. In general, ATP release is directed apically. However, there may be physiological and pathophysiological exceptions to this rule as well (macula densa of the kidney as an example, see below). The mixture of P2Y, P2X, and P1 receptors in the apical and basolateral membrane domains may also differ. Nevertheless, a given epithelial cell commonly expresses all three purinergic receptor subfamilies and, often, multiple P2Y, P2X, and P1 receptor subtypes (see also below). That mixture of purinergic receptor subtypes governs what nature of signal is transduced from the released ATP and its metabolites, either in the luminal (apical) or interstitial (basolateral) microenvironments.

### 7.1. Lung and airway epithelium

There are two general sites of ATP release and signaling in the human lung and airways: the airway lumen and the submucosal gland secretions (Fig. 7). Although it has not been investigated to date, ATP secretion in the distal lung at the gas exchange zone may also occur with surfactant secretion. Airway surface epithelium in the smaller and larger conducting airways is capable of ATP and UTP release [164] in response to a myriad of stimuli (flow, touch, cyclic nucleotides, hypotonicity, and calcium agonists); moreover, secretions from submucosal glands that lie beneath the pseudostratified surface epithelium may also supply autocrine nucleotides and nucleosides. Neuroendocrine cells, mast cells, and goblet cells along the airway may also release ATP along with other agonists from their granules. The analogy of the submucosal gland secreting purinergic agonists with mucins and other substances can be extended to the hepatic and pancreatic acini and the secretory glands/coils of the sweat gland and the salivary gland.

With regard to microenvironments, however, the airway surface liquid (ASL) bathing the cilia on ciliated airways

epithelium in the large conducting airways is a microenvironment of interest in physiological paradigms and in pathophysiological contexts such as cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) [165,166]. Because purinergic signaling governs ciliary beat, epithelial cell signaling, and epithelial solute and water transport, extracellular nucleotide and nucleoside signaling in this microenvironment may be essential to regulating the composition of the ASL and the function of this critical extracellular space. Several laboratories, including our own, have found a loss of purinergic signaling in CF epithelial and heterologous cell models lacking CFTR [17]; as such, loss of purinergic signaling may be critical to the loss of CFTR function, loss of ciliary beat, abnormal ASL composition, and overall loss of mucociliary clearance. These issues will be revisited below.

As agonists were being screened to restore chloride and fluid secretion in CF airways epithelium, nucleotide agonists emerged quickly as chloride and fluid secretagogues that stimulated chloride and fluid secretion independent of CFTR. Importantly, Knowles et al. [167] showed that extracellular nucleotides stimulated chloride secretion in human patients with CF, triggering interest in targeting any and all purinergic receptors for CF therapy. Importantly, purinergic agonists in addition to ATP such as UTP, UDP, and ADP (as well as poorly hydrolyzable analogs of those listed) were also efficacious in CF as well as non-CF airway epithelial model systems [168,169]. This led to the identification of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> receptors in human airway epithelial model systems [170]. In addition, adenosine receptors (the P1 receptors) also stimulated chloride secretion in airway epithelial cells [171]; however, the major adenosine receptor, the A<sub>2</sub> receptor, activates the cAMP/PKA signal transduction cascade and, eventually, CFTR. It is dysfunctional in CF. Several other laboratories have confirmed as well as extended these early findings in human epithelial cells or cells from other species [172–175].

Our laboratory has added work showing abundant expression and function of the P2X purinergic receptor channels in epithelia [141]. Biochemical characterization of P2X<sub>4</sub> and P2X<sub>5</sub> in human airway epithelial model systems with subtype-specific antibodies is currently in progress in our laboratory. We also cannot rule out the possible expression of P2X<sub>6</sub>, which is not detected by our degenerate RT-PCR primers and for which antibodies are not available. A unifying theme in this chapter will be the fact that most, if not all, epithelial cell models express both P2Y G protein-coupled receptors and P2X receptor channels, sometimes in the same membrane domain of the polarized epithelium (Fig. 7). Because each class of receptor increases cytosolic calcium, albeit via different mechanisms, both receptor subtypes are viable targets for pharmacotherapy of CF in the lung and airways (see below).

Both P2Y G protein-coupled receptors, via phospholipase-induced release of  $\text{Ca}^{2+}$  from intracellular stores, and

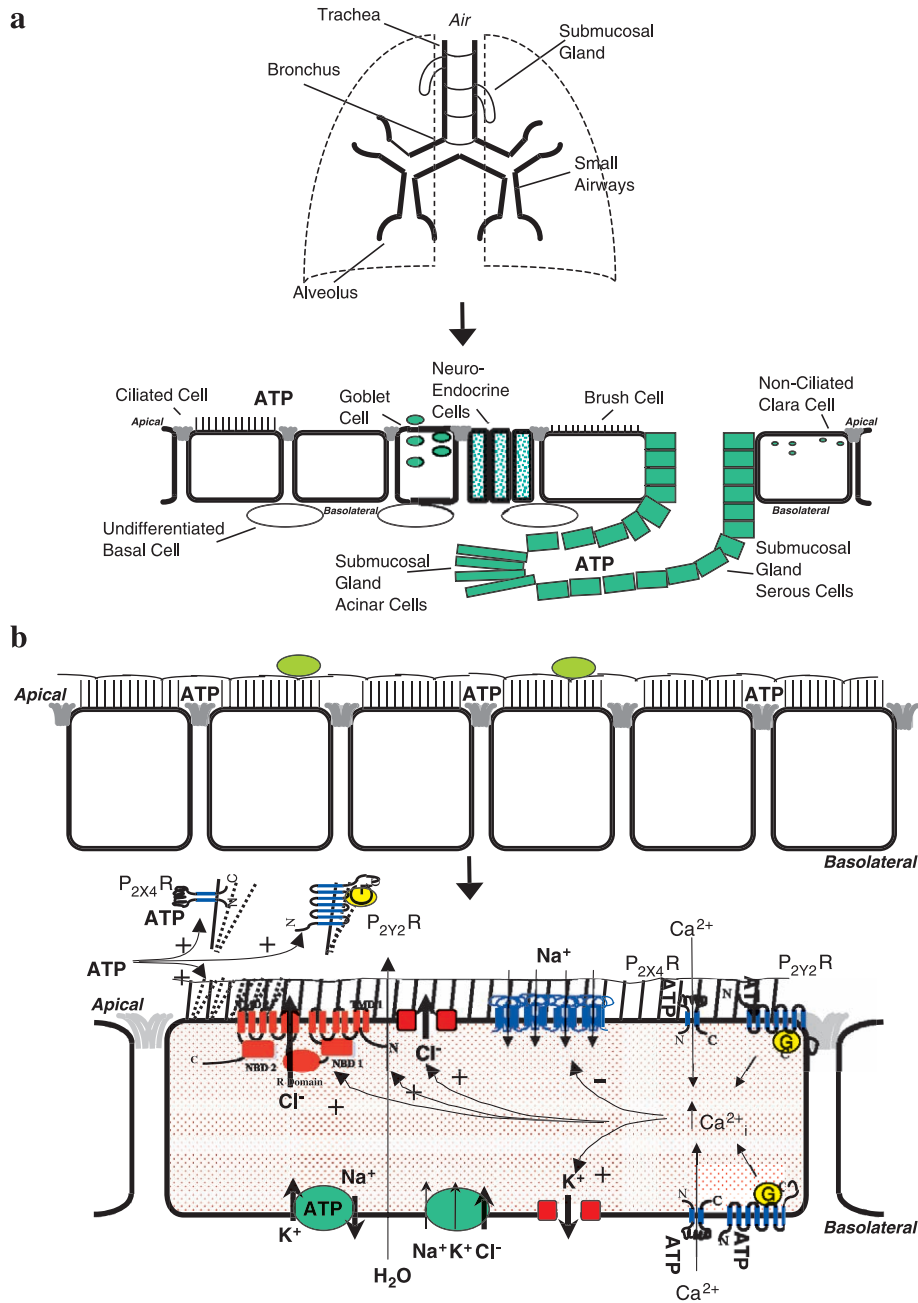


Fig. 7. Nucleotide signaling in the airways epithelium. Schematics of the whole lung and airways, a cross-section of a large airway with the many different subtypes on the surface and a submucosal gland beneath, a multi-ciliated airways epithelium segment of a large airway, and a single airway epithelial cell showing just some of the many functions that autocrine or paracrine purinergic signaling modulates. This regulation breaks down in CF (see text for details).

P2X receptor channels, via direct influx of  $\text{Ca}^{2+}$  through the channel from extracellular stores are capable of triggering calcium-dependent signal transduction cascades. P2Y-mediated effects on  $\text{Ca}^{2+}_i$  have been studied extensively in human airway epithelial cell models by Paradiso and others [176]. Zsembery et al. [154], using an IB3-1 CF human airway epithelial cell model for therapeutic implications in CF airway, have shown that both P2Y and P2X subtypes increase cytosolic calcium. Intriguingly, ATP stimulates both subtypes, although a fully transient increase in calcium is triggered by P2Y receptors, while P2X receptors cause a

sustained increase in  $\text{Ca}^{2+}_i$  (perhaps through the P2XR channels themselves and/or by opening store-operated or “Transient Receptor Polarization (TRP)” calcium channels). P2X receptor channel-mediated increases in cytosolic calcium are greatly potentiated by removal of extracellular  $\text{Na}^+$  by replacement with NMDG [154]. We interpret this result to suggest that  $\text{Na}^+$  competes with  $\text{Ca}^{2+}$  as each cation passes through the ATP-gated pore (each cation has a favorable gradient for entry). The P2X receptor-induced calcium influx is prevented by EGTA and augmented by raising extracellular  $\text{Ca}^{2+}$  [154].



Together, P2X and P2Y receptor activation leads to a pronounced, additive, and sustained increase in  $\text{Ca}^{2+}_i$ . This fact is critical for several reasons. First, either or both receptors could be targeted to trigger calcium-induced chloride secretion and potassium channel opening, which would be beneficial to the CF epithelium. Second, both receptors could be targeted to suppress absorptive  $\text{Na}^+$  channels by calcium-dependent mechanisms. Such effects on all of the above would also correct abnormal cell volume regulation in the CF airways epithelium (see below). Local increases in calcium also augment ciliary beat frequency (see below), which would benefit attenuated mucociliary clearance in CF. Targeting either or both ATP receptor subfamilies for pharmacological therapy of the CF lung and airways is logical and feasible and takes full advantage of naturally expressed membrane proteins that are largely apical but are also basolateral.

Early work from Knowles et al. [167,171] showed that purinergic agonists stimulated chloride secretion in CF as well as non-CF airway epithelial model systems. The most important demonstration of this was in *in vivo* nasal potential difference (PD) measurements in CF patients [167]. Jiang et al. [177] subsequently showed that purinergic agonists stimulated fluid secretion in CF and non-CF airway epithelial cells. Purinergic agonists emerged from a “kitchen sink” approach of adding a large panel of agonists to CF epithelial model systems to identify which agonist, if any, could stimulate chloride secretion independent of CFTR. Many studies confirmed this work in other airway epithelial model systems from human and other species. Guggino et al. showed that purinergic receptors stimulated multiple types of chloride channels, including CFTR and the ORCCs from both the apical and basolateral membranes of primary rat tracheal epithelial cell monolayers [169]. It was concluded that the  $\text{P2Y}_2$  receptor was critical to the apical-mediated stimulation of chloride secretion; however, rank order potency pharmacology most consistent with the  $\text{P2Y}_3$  receptor (a species homolog to  $\text{P2Y}_1$ ) stimulated chloride secretion from the basolateral side. As mentioned, UDP and ADP also stimulated chloride secretion in airway epithelia.

Epithelial cells release ATP in response to hypotonicity or dilution of the extracellular osmolality [17,19,21,47,51,178]. Hypotonicity-induced ATP release is potentiated by the expression of CFTR in the epithelial cell and is dampened when CFTR is absent from the plasma membrane [17,19]. Because CFTR is a regulator of other ion channels, transporters, and vesicle trafficking and fusion, CFTR may augment ATP release by positively regulating any and all of these ATP release mechanisms [17].

More recently, our laboratory has shown that CF human airway epithelial cells are sluggish in their recovery from a hypotonic insult, a process known as regulatory volume decrease or RVD [17]. Transient or stable transfection of the wild-type CFTR cDNA into CF human airway epithelial cells corrects defective cell volume recovery in response to hypotonicity. Wild-type CFTR modulation of human airway

epithelial cell volume regulation is blocked by the ATP scavengers, hexokinase or apyrase, and the global P2 receptor antagonist, suramin. Taken together, these data suggest that the presence of CFTR in the plasma membrane and CFTR-dependent ATP release govern epithelial cell RVD. This result begged the question: would addition of selective purinergic agonists to  $\text{P2Y}$  and  $\text{P2X}$  receptor subtypes rescue defective volume regulation in CF human airway epithelial cells. This is indeed the case. Both  $\text{P2X}$  receptors and  $\text{P2Y}$  receptors stimulate chloride and, likely, potassium efflux from epithelial cell during RVD. This is one critical answer to the question: why would a particular epithelial cell model express both  $\text{P2Y}$  and  $\text{P2X}$  receptors in the same cell? One answer is for protective redundancy against osmotic or other environmental insults (anoxia and ischemia, for example). Because epithelial cells are exposed to very different and dynamic microenvironments on their apical and basolateral sides, epithelial cells may express  $\text{P2Y}$  and  $\text{P2X}$  receptors in both membrane domains for this protective redundancy. A similar paradigm is evolving in hepatocytes and cholangiocytes in work by Feranchak et al. [47,178]. Another answer is simply that  $\text{P2X}$  receptors mediate fast and sustained increases in calcium signaling, while  $\text{P2Y}$  receptors mediate slower and only transient increases in cytosolic calcium.

In addition to stimulating  $\text{Cl}^-$  and  $\text{K}^+$  efflux from the epithelial cell, ATP (and UTP) have also been observed to inhibit  $\text{Na}^+$  influx pathways. Devor and Pilewski [179] have shown that UTP, by binding to purinergic receptors and increasing intracellular calcium concentrations, leads to a long-term inhibition of  $\text{Na}^+$  entry to the cell from the apical media. This was studied in cells with normal and mutant forms of CFTR. They conclude that nucleotides (ATP and/or UTP) could have a dual therapeutic role in the airway, stimulating  $\text{Cl}^-$  efflux and inhibiting  $\text{Na}^+$  entry. This conclusion has been borne out by a handful of studies in airway and other miscellaneous epithelial cell models, showing that stimulation of purinergic receptors stimulates  $\text{Cl}^-$  secretion while, at the same time, attenuating  $\text{Na}^+$  absorption. Inglis et al. [180] studied these effects in distal bronchi and in rat fetal distal lung epithelial cells [181]. Iwase et al. [182] showed similar results in rabbit tracheal epithelium. While these were different preparation of distal airway epithelial cells, the conclusions were similar. ATP and UTP evoked a transient stimulation of  $\text{Cl}^-$  secretion and a sustained inhibition of  $\text{Na}^+$  absorption in the polarized monolayers of distal airway epithelia. In the nasal PD measurements of porcine distal bronchi, transepithelial PD was measured in cannulated and perfused preparations. UTP hyperpolarized the PD transiently; this stimulation was inhibited by serosal bumetanide. PD then declined to a sustained level lower than basal; this sustained inhibition was prevented by luminal amiloride. Because thapsigargin failed to inhibit UTP-stimulated  $\text{Cl}^-$  secretion but did block the inhibition of  $\text{Na}^+$  absorption, these results suggest that UTP stimulates  $\text{Cl}^-$  secretion by a calcium-independent mechanism, while

UTP inhibits  $\text{Na}^+$  absorption via  $\text{Ca}^{2+}_i$ . Nevertheless, Inglis et al. argue that both effects of extracellular nucleotides should promote hydration of the airway.

The mucociliary system in the airway is necessary for optimal removal of mucus and inhaled pathogens and particles. Cilia on the surface of the airways epithelium beat at regular intervals and in a uniform direction toward the pharynx, and this facilitates the movement of materials along and up the “mucociliary escalator.” A key stimulus for enhancing ciliary beat is increased intracellular  $\text{Ca}^{2+}$  concentrations within the cilia themselves. If  $\text{Ca}^{2+}$  influx is impaired, this could lead to jeopardized mucociliary clearance and disease. Of the hormones and neurotransmitters that are known to stimulate ciliary beat, the most potent is ATP. ATP acts primarily in this system by stimulating  $\text{Ca}^{2+}$  influx from the external milieu, as well as stimulating internal stores to release  $\text{Ca}^{2+}$ . Ma et al. [183] have shown that extracellular sodium ions actually inhibit an ATP-gated calcium channel, perhaps through competition of both cations for the ATP-gated pore.  $\text{Na}^+$ , therefore, attenuates ATP-dependent ciliary beat. Their findings suggest a physiologically significant relationship between  $\text{Na}^+$  concentrations and ciliary beat. This has potentially significant therapeutic implications, suggesting that decreasing airway surface fluid  $\text{Na}^+$ , while increasing  $\text{Ca}^{2+}$  in this fluid, may augment calcium influx into the cilium via P2X receptors and could increase mucociliary clearance in such diseases as cystic fibrosis, primary ciliary dyskinesia, or chronic bronchitis (Fig. 7).

### 7.2. Cystic fibrosis: a loss of extracellular purinergic signaling in the lung airways and in other tissues

Our laboratory as well as other laboratories have documented a role for CFTR and other ABC transporters in governing ATP release and autocrine and paracrine purinergic signaling. In the disease, cystic fibrosis (CF), purinergic signaling is attenuated. We believe that this defect, along with the primary defect in  $\text{Cl}^-$  channel function and  $\text{Cl}^-$  permeability [184], are major contributing factors to the abnormalities in the CF airway and, possibly, in other tissues affected in CF.

The CFTR protein is widely believed to form an anion channel in the apical membrane, but there is speculation as to exactly what it transports. There is a large amount of evidence that CFTR is a  $\text{Cl}^-$  channel activated by cAMP. Some believe that CFTR also has the capacity to conduct ATP [11], but this is very controversial [13]. Past and current research is focusing on a separate ATP conductance channel that is closely associated with the CFTR protein [17,18]. Because CFTR also regulates numerous other processes in the airway epithelial cell, CFTR may regulate ATP release, mediated by ATP channels and ATP-filled vesicles, to accomplish these other regulatory functions.

It has been shown that CFTR is necessary for extracellular nucleotide signaling. In CF epithelial cells that lack

functional CFTR at the apical membrane, nucleotide signaling is absent or insufficient. Therefore, the physiological consequences of this lost signal must be addressed. There is a loss of chloride conductance that may be necessary for the chloride secretion involved in RVD and transepithelial transport [12,167]. There could also be a loss of potassium and fluid secretion due to the loss of nucleotide signaling, two processes also critical in RVD. These consequences could lead to impaired intracellular cell volume regulation. An impairment in intracellular volume control, due to an inability of the epithelial cell to “sense” its external osmotic environment and/or to regulate its own volume, may also directly impact upon the abnormal ASL microenvironment observed in CF. Smith et al. [185] have observed a curious increase in ASL ionic strength in CF epithelia when compared to non-CF cells. This would also increase the tonicity of this microenvironment. Matsui et al. [186] observed a reduced ASL depth and volume in CF epithelia, but no difference in ionic strength. Zhang and Engelhardt [187], using their elegant xenograft model of well-differentiated non-CF and CF epithelia, showed defects in ionic strength and volume in the CF ASL versus the normal counterpart. These results and hypotheses relate back to an older and unappreciated study by Valverde et al. [188], who observed defective cell volume regulation in the intestinal crypts of CF knockout mice when compared to wild-type mice. The possibility that these defects in ASL composition and cell volume regulation may be caused by defects in autocrine ATP release and signaling remains to be determined.

## 8. Epithelial cells along the renal nephron

Once filtered at the glomerulus or secreted into the lumen of the nephron (proximal tubule cells release micromolar quantities of ATP) [21,44,189], the ATP is trapped in the tubular lumen as a signaling molecule. It is then propelled downstream by the tubular fluid rapidly and robustly where it can act on downstream tubules in a paracrine manner. Cells derived from multiple nephron segments express multiple P2Y, P2X, and P1 receptors, and Leipziger et al. and Satlin et al. [190–192] have shown the presence of luminal P2 receptors in isolated and perfused cortical collecting tubule or duct (CCT or CCD) preparations from mouse and rabbit. Even once that ATP is excreted in the final urine and exits the nephron, it can signal the uroepithelium (or urothelium) in the ureter and urinary bladder. Moreover, the urothelium releases ATP in response to increased distension of the ureter, the bladder, and associated structures [193]. Taken together, the lumen of the nephron and beyond provide an ideal microenvironment with a fluid medium that can transmit this autocrine and paracrine signaling molecule (Fig. 8).

The process of tubuloglomerular (TG) feedback, where changes in the luminal content of salt and water is sensed by

the small plaque of macula densa (MD) cells in the cTAL and where that sensation is transduced to the glomerulus of the same nephron, is widely accepted but poorly understood. A long-held hypothesis is that a local mediator or paracrine agonist may be released by MD cells and regulate vascular tone of the glomerulus by causing contraction of glomerular mesangial cells and constriction of the afferent arteriole of that glomerulus. Past work has suggested that adenosine may be that mediator. Through an elegant, freshly dissected cTAL/MD/glomerular preparation, Liu et al. [30] are examining TG feedback signaling mechanisms more directly. Briefly, they have found that changes in perfused NaCl concentrations are sensed in the lumen of the cTAL by the specialized MD cells, leading to ATP release from the basolateral surface of the MD plaque. ATP or its metabolites (adenosine, in particular) would then bind to glomerular mesangial cells in the interstitium and, ultimately, vascular smooth muscle cells in the afferent arteriole of the glomerulus. Via P2Y and/or P2X receptor channels, ATP would elevate cytosolic  $\text{Ca}^{2+}$  in both cell types, leading to constriction of the afferent arteriole and a decrease in renal blood flow and glomerular filtration rate to that single glomerulus. Adenosine could do the same via its A1 receptor. In short, it is classic paradigm for paracrine purinergic signaling involving specialized renal epithelial cells and their “cross-talk” with other renal glomerular cell types.

### 8.1. Collecting duct

McCoy et al. [194] studied a mouse IMCD cell line (mIMCD-K2) to determine if nucleotides regulated NaCl

transport and, if so, which purinergic receptor subtypes were involved. They observed that ATP,  $\alpha\beta$ -methylene ATP and UTP stimulated  $\text{Cl}^-$  secretion and inhibited  $\text{Na}^+$  absorption. These P2 agonists as well as specific and degenerate RT-PCR implicated four receptor subtypes: P2X3, P2X4, P2Y1, and P2Y2. Further analysis showed that apical nucleotide receptors were more effective in inhibiting  $\text{Na}^+$  absorption and stimulating  $\text{Cl}^-$  secretion. Leipziger et al. have found similar results in M1-CCD cells and MDCK cells, and Deetjen and Leipziger and, later, Satlin et al. [190–192,195,196] found an apical P2Y2 receptor in isolated, perfused CCDs that increases cytosolic  $\text{Ca}^{2+}$  in these tubules. Similar modulation of ion transport in this segment is likely. Purinergic receptors also modulate water transport in the collecting duct. Kishore and Knepper found that P2Y2 receptors were expressed along the nephron in general and in renal medulla in particular. ATP and UTP, via this receptor, inhibited vasopressin-induced water permeability across the renal collecting duct. This paradigm is described in detail in a recent review [56]. Fig. 8 shows a collecting duct epithelial cell model illustrating this purinergic regulation in this nephron segment.

### 8.2. Autosomal dominant polycystic kidney disease: a change in nephron structure creates a detrimental autocrine signaling environment

Purinergic receptors and signaling are also critical along the nephron of the kidney. The richest source of ATP release in the nephron is the proximal tubule. There are many reasons why this is the case, including an accelerated

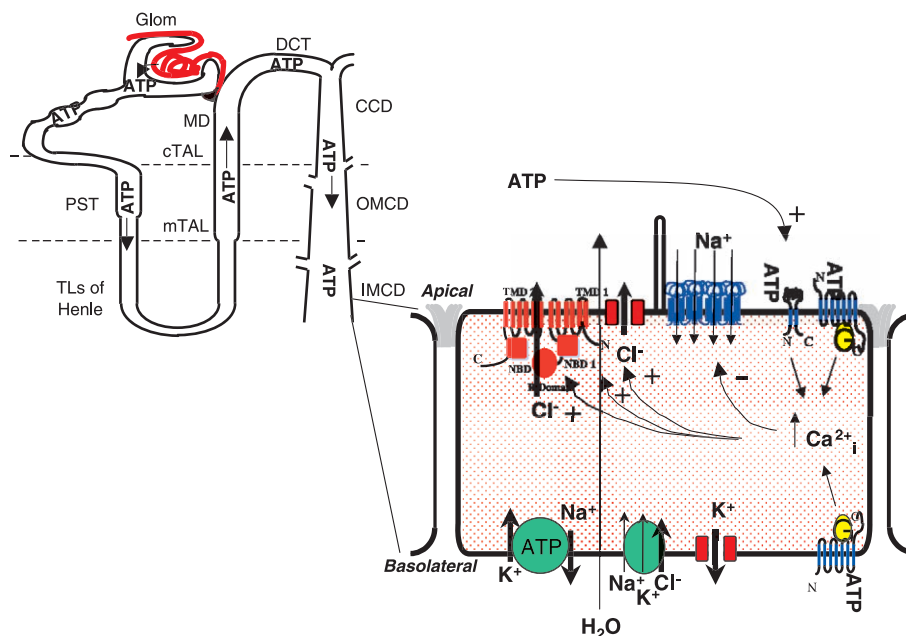


Fig. 8. Collecting duct epithelium in the context of the nephron. Schematic of the whole nephron and a cartoon of nucleotide regulation of a mono-ciliated collecting duct cell. Despite some similar modulation of NaCl and water transport, P2XRs have only been shown functionally to be present on the apical membrane on renal epithelia.

metabolic and transport rate in the proximal tubule and/or the expression of multiple ABC transporters like CFTR, mdr, and MOAT. Once released into the tubular lumen, ATP would be trapped as a charged anionic species and carried in the tubular fluid downstream to the other nephron segments, where it can bind to and interact with purinergic receptors. Autocrine and paracrine ATP signaling in the tubular fluid via purinergic receptors can affect any number of renal functions, due to the versatility of purinergic control over epithelial functions and signaling pathways (see recent reviews, Refs. [56,189]).

Because purinergic signaling plays an autocrine and paracrine role in the regulation of renal tubular function, defects in purinergic signaling could contribute significantly to kidney pathophysiology. Extracellular purinergic signaling is known to modulate water and salt balance along the nephron. Extracellular ATP in the apical environment or tubular lumen has been shown to inhibit water and salt reabsorption by the kidney. Therefore, the addition of purinergic agonists has been proposed to be of therapeutic benefit to decrease water and salt reabsorption, thus decreasing blood volume and pressure. A recent review examined this paradigm in some detail [56].

The relationship between autocrine and paracrine purinergic signaling and the renal disorder, PKD, is not intuitively obvious. However, PKD is a progressive disorder characterized mainly from the slow and gradual remodeling of the tubules of the kidney and the ducts of the pancreas and liver. In autosomal dominant PKD (ADPKD), this remodeling results in the formation of a fluid-filled cyst, created from the closure of two ends of a renal tubule. In autosomal recessive PKD (ARPKD), the tubules and ducts dilate; however, they only form cyst-like structures in end-stage disease that never fully close off. Either way, an abnormal microenvironment encapsulated by a single monolayer of epithelial cells develops. As alluded to above and in other reviews, such a microenvironment is ideal for autocrine and paracrine signaling [197].

Our laboratory has shown that ATP (and, likely, its metabolites) are present in micromolar quantities in a subset of ADPKD cyst fluid samples [21]. In this study and in another very recent one [44], ATP release was found to be as or more robust in PKD epithelia versus normal controls. This could be due to increased metabolism and/or proliferation of PKD epithelia during their reversion to an undifferentiated phenotype. More recent work has shown that both P2Y and P2X receptors are present on the luminal membrane of human ADPKD monolayers that stimulate  $\text{Cl}^-$  secretion by a cytosolic calcium-driven mechanism. As such, all of the elements are present for autocrine and paracrine ATP and ATP metabolite signaling to drive  $\text{Cl}^-$  secretion into the cyst. Obviously, this would be detrimental to the progression of ADPKD. An additional problematic feature of “trapped” purinergic signaling within the cyst is the fact that purinergic agonists are mitogens or co-mitogens along with growth factors for renal epithelial cells and

mesangial cells [198–200]. In PKD, growth factors are also released instead into the cyst. There, they interact with growth factor receptors mislocalized to the luminal membrane, leading to a devastating positive feedback loop for growth and proliferation of the cysts [201,202]. The autosomal dominant form of PKD (ADPKD) results in distinct cysts, or fluid-filled spheres lined by epithelial cells. Any normally apical ion or fluid transport is instead released into the encapsulated cyst. Therefore, any secretagogue could prove to be detrimental. Growth factors are normally released apically from the cell. Therefore, in some cell models, purinergic agonists were observed as mitogens or co-mitogens with growth factors. Therefore, ATP release into the cyst lumen could further prove to be detrimental.

## 9. Hair cell epithelium of the inner ear

The hair cells that line the cochlea of the inner ear respond to sound waves that enter the ear. ATP has been shown to be present in the endolymph, the fluid medium of the inner ear that bathes the hair cells [203]. Moreover, P2X purinergic receptor channels are present on the stereocilia that lie atop each hair cell [204]; the cilia bend in response to each sound wave and the mechanical changes in the cilia are transduced from a mechanical signal to an electrical one. Purinergic signaling is emerging as a critical process in sound transduction by the cilia on the hair cell [203]. Not only could ATP be released from the hair cells but that ATP could bind to the P2X receptors on the cilia to augment ciliary beat.

ATP is a critical autocrine and paracrine agonist in the endolymph and the scala media that fill the organs of the inner ear. P2 receptors have been found throughout the specialized epithelial cells and cell membranes that are present in the cochlear and vestibular system. This topic has been reviewed recently by Housley [203]. Basal levels of ATP in the perilymph and endolymph are nanomolar, and they increase in response to noise stress, sound waves, and hypoxia. Ecto-ATPases and ecto-apyrases may suppress the levels of extracellular ATP; however, the full range of stimuli for ATP release may not be appreciated and increases in ATP release (surrounding the stereocilia of the hair cells, for example) may not be readily detectable. Housley cites that the sources of released ATP are not appreciated and present an important future direction of this work. However, a recent study by Munoz et al. [205] showed that stores of ATP located in vesicles within marginal cells of the stria vascularis were released to increase ATP in the endolymph significantly following a sound stimulus. In contrast, hypoxia did not promote a statistically significant increase in endolymph ATP, although a slight increase was noted. It was also noted that substantial ATP was present under basal conditions, suggesting a role in the maintenance of inner ear and cochlear and vestibular function.



Despite continuing work on the sources of released ATP, it is known that extracellular ATP modulates transduction of sound waves by action of P2X receptors on the stereocilia and by P2Y receptors there and elsewhere. This is reviewed elegantly by Housley [203]. It is thought that P2X<sub>2</sub> receptors function together with stretch-activated non-selective cation channels in the stereocilia to depolarize the outer hair cell membrane, suggesting that ATP may play a significant role in the inner ear signaling transduction pathway [124,206,207]. However, it is possible that additional P2X receptor subtypes may contribute to the phenotype in particular inner ear cell types. P2X receptors also play critical roles in Reissner's membrane and in intermediate cells of the stria vascularis, where they mediate K<sup>+</sup> shunt conductances [208]. It could be argued that the P2X receptor channels and other secretory K<sup>+</sup> channels contribute to the high K<sup>+</sup> concentrations in perilymph and endolymph. Indeed, mutation of the KvLQT channel in the stria vascularis marginal cell causes deafness in patients that also have long QT syndrome of the cardiac myocyte. The P2Y<sub>4</sub> and P2Y<sub>2</sub> receptors also play a critical role in regulating this KvLQT and other KCNE channels in strial marginal cells and vestibular dark cells [209,210]. Deeper in the inner ear, Marcus et al. have also found a role for P2X<sub>2</sub> receptors in parasensory cation absorption by cochlear outer sulcus cells and vestibular transitional cells [204]. In fact, guinea pigs with vestibular imbalance disorder were corrected by infusion of ATP and other purinergic agonists into the scala tympani.

Purinergic signaling also affects other inner ear epithelial cell functions. Hensen's cells of the cochlea, specialized epithelial cells in the inner ear, respond to extracellular ATP with changes in ionic currents and concomitant increases in Ca<sup>2+</sup><sub>i</sub> [211]. Ionic currents that were activated included a rapid inward current, a more slowly rising inward current, and a slowly developing reduction in input conductance. ATP also appeared to inhibit cell–cell communication via gap junctions. The fast current activated by ATP were the P2X receptor channels located on these and other specialized sensory epithelial cells in the cochlea, while P2Y receptors mediated the slow responses in ionic currents and Ca<sup>2+</sup><sub>i</sub>. The same group also performed similar work on Dieter's cells and showed that ATP promoted gap junctional communication between cells. Unfortunately, Fura-2 imaging of multiple cells within the tissue for Hensen's cells or Dieter's cells was not performed to monitor Ca<sup>2+</sup> wave propagation. The inner ear and its specialized epithelial cells designed to sense and capture sound is a rich ground to study autocrine and paracrine nucleotide signaling. Taken together, there are profound physiological roles for extracellular ATP signaling and both P2X and P2Y receptors in the specialized ciliated and non-ciliated epithelial cells of the inner ear. Luciferase-based or other detection methods need to be applied to freshly dissected, endolymph-filled inner ear preparations to gauge the role of extracellular ATP in an *in vivo*-like preparation.

## 10. Future directions in the context of unanswered questions

### 10.1. Why does a specific cell need P2Y and P2X receptors?

There are answers to this question already; however, there will likely be many more. P2X receptors that allow calcium influx directly are thought to be the fast-acting purinergic receptor, while P2Y receptor mediate slower responses. Having said that, both increase cytosolic calcium within seconds; however, P2X receptors mediate sustained calcium increases in epithelial cells. Thus, a cell may indeed need both, and there is considerable evidence that neurons and epithelial cells express both subfamilies. Our laboratory has shown that both P2Y and P2X receptors are expressed functionally in the apical membrane of airway and kidney epithelia and vascular endothelia *in vitro* and *in vivo* [45,46,142,195]. Another reason may be for redundancy to protect against osmotic insult, anoxia or hypoxia, or other forms of cell injury. Or, related to the next question, a cell may need both for basal maintenance of essential functions and cell signaling. Nevertheless, study of the native P2X and P2Y receptors in these and other cells is becoming a necessity.

### 10.2. Does ATP release and signaling within microenvironments govern basal cell function and signaling?

Evidence is emerging that constitutive ATP release occurs under basal conditions to maintain physiological “setpoints” for signaling and function. Ostrom et al. [212] showed in Madin–Darby canine kidney (MDCK) cells that phosphatidylinositol turnover and arachidonic acid metabolism were altered when autocrine purinergic signaling was abolished. Our laboratory has published evidence that basal cytosolic calcium is modulated by endogenous ATP release and signaling in epithelial cells [46]. We also have preliminary evidence that endogenous ATP release and signaling modulates cell volume regulation under isotonic conditions and ciliary beat in airway and other preparations. Indeed, in past cardiovascular paradigms, ATP and adenosine have been characterized definitively as local metabolites that modulate vascular tone. Again, these studies can only be done in native cells and confirmed with heterologous cell systems that are carefully handled.

### 10.3. Is signaling emanating from P2R activation only dependent on calcium?

The answer to this question is likely to be complex. Sustained increases in cytosolic free calcium derived from P2XRs and extracellular stores may trigger calcium-dependent protein kinases (CaCMKs, Pyk2, ERKs, PKC, etc.) which may have many additional targets within the cell and affect cell function in limitless ways. Some or all of these calcium-dependent protein kinases affect the activity of

transcription factors and, thus, gene expression. Evidence is accumulating more slowly that P2YRs may couple to other signaling effector enzymes besides phospholipase C $\beta$ , including PLA $_2$  and PLD. Native cells will likely aid this work and make it more physiologically relevant. Moreover, care must be taken to rule out or account for endogenous ATP release and signaling from the cell model or tissue preparation itself that may influence signaling cascades independent of exogenously applied agonists.

#### 10.4. What is the stoichiometry of a P2XR multimer?

This is a ticklish question that is difficult to define or characterize. There is controversy over this question in the ENaC field as well as in every other ion channel field. Because IRK channels likely form tetramers, it is assumed that ENaCs and P2XRs may do the same. However, the large extracellular domain that these subfamilies share and is lacking in the IRK family may confound this multimeric assembly. There is no doubt that multimerization is critical for function; however, a consensus on stoichiometry would help understand P2XR expression and function better.

#### 10.5. Are P2XRs targets for therapy of different diseases?

Exogenous ATP has been shown to correct hearing disorders. As such, it is possible that targeting of P2X $_2$  on stereocilia may be worth pursuing. P2XRs are a rich and important target for therapy of CF. In airway epithelia, multiple P2XR subtypes are expressed on both apical and basolateral membrane domains. In vivo nasal PD measurements showed a stimulation of chloride transport in CF knockout mice as well as normal mice when the agonists were added luminally. Current work has returned to this and other assays with different conditions designed to maximize P2XR activity and the sustained calcium increase that is mediated by P2XRs. More importantly, the P2XRs expressed in epithelia desensitize poorly (P2X $_2$ , P2X $_4$ , P2X $_5$ , and, possibly, P2X $_6$ ); the same cannot be said for G protein-coupled receptors like P2Y $_2$  targeted by other groups for CF therapy. P2XRs, like P2Y $_2$ , would restore chloride and fluid secretion and dampen sodium absorption but without desensitization and with a sustained increase in cytosolic calcium. This would correct abnormal cell volume regulation. Possibly, the most important effect would be to amplify ciliary beat frequency. Application of P2XR agonists would also require modified conditions of the saline in which it was nebulized and aerosolized to maximize its effect. Our laboratory is ardently pursuing this therapeutic approach.

On the other hand, inhibition of P2XRs and, possibly, other calcium entry channels may be beneficial for therapy of ADPKD. This could suppress chloride and fluid secretion and fluid accumulation as well as the enhanced proliferation rate of the monolayers of renal epithelial cells that encapsulates the cyst, two hallmarks that enhance the progression of the disease after cyst formation. At the same time,

inhibition of calcium entry might augment ENaC-mediated sodium and fluid absorption, causing the cysts to shrink. Our laboratory is pursuing this therapeutic hypothesis.

These questions are just a few of the many that drive those of us who are obsessed “purinergics.”

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