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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$^-$) 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 $P2X_4$ 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 $P2X_4$ 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),[1] 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 ($[Ca^{2+}]_i$) potently 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 $P2Y_2$ 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 Cβ) 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$^-$) (31) human airway epithelial cell models, to dissect out P2X-specific and P2Y-specific mechanisms of trigger-

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[1] 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; ADPβS, adenosine 5′-[β-thio]diphosphate; 2MeSATP, 2-methylthio ATP; α,β-meATP, methylene ATP; BzBzATP, benzoyl-benzoyl-ATP; 2APB, 2-amino-ethoxyliphenyl borate.

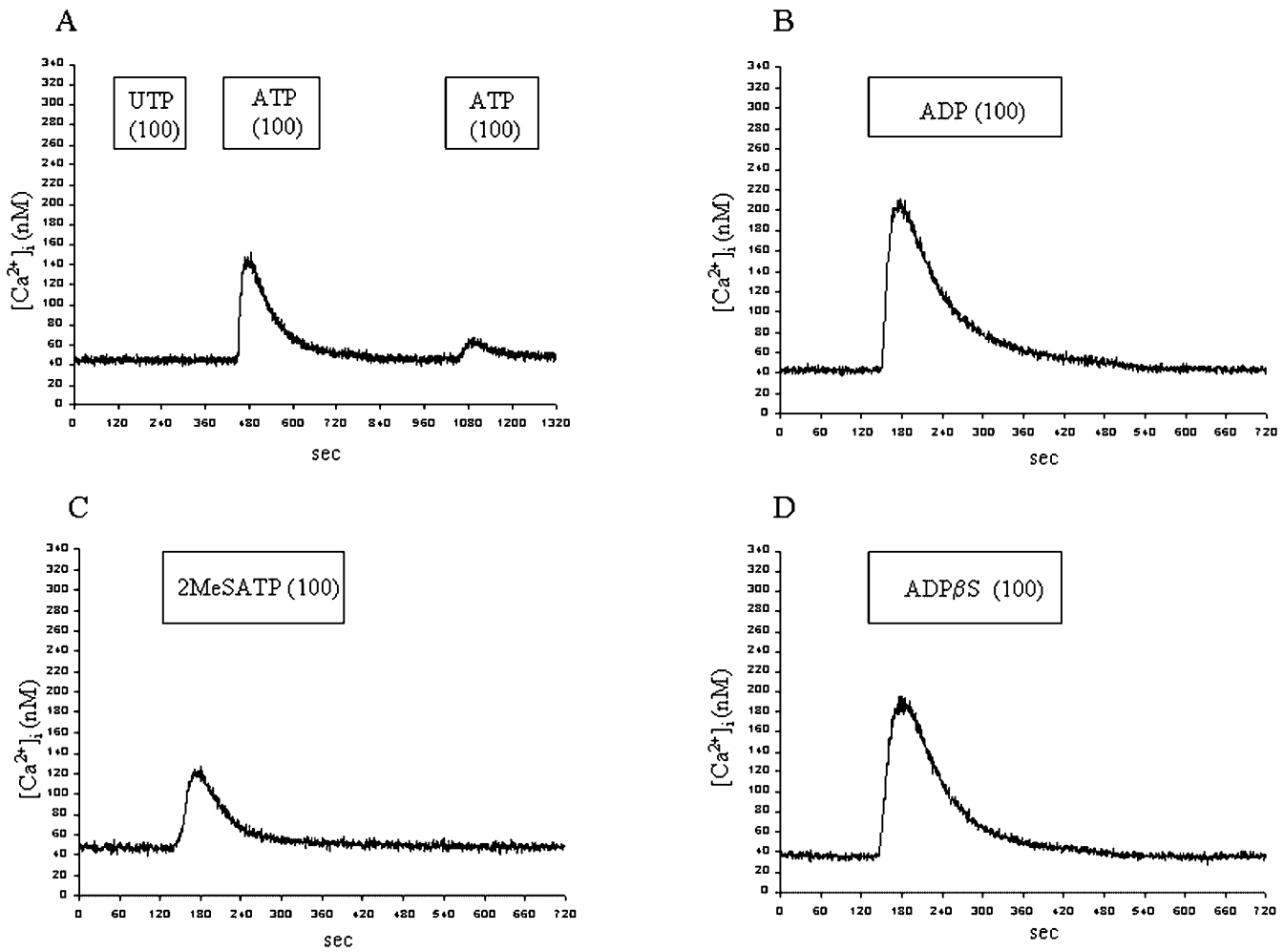

FIG. 1. **Original traces showing the effects of ATP and UTP (100 μM each) (*A*), ADP (100 μM) (*B*), 2MeSATP (100 μM) (*C*), and ADPβS (100 μM) (*D*) on $[Ca^{2+}]_i$.** IB3-1 cells were superfused with Na^+-containing medium (solution A). *A,* please note that the second application of ATP was without 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 $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ that is sustained and prolonged. Results described herein, using Fura-2/AM-based imaging, show that activation of P2Y and P2X receptors increases $[Ca^{2+}]_i$ by completely distinct mechanisms. P2Y receptors elicit a transient increase in $[Ca^{2+}]_i$ derived from intracellular endoplasmic reticulum (ER) stores, whereas P2X receptors trigger a sustained rise in $[Ca^{2+}]_i$, allowing Ca^{2+} influx from the extracellular space. In addition, biochemical evidence shows that the $P2X_4$ receptor is the major epithelial subtype present in both cell lines. Thus, we conclude that epithelial P2X receptors function as ATP-gated 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 (ΔPhe-508) and a premature stop codon mutation (W1282*X*) (30). 16HBE14o$^-$ 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% 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× L-glutamine (Invitrogen), and 1.25 μg/ml Fungizone (Invitrogen). 16HBE14o$^-$ 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 Ca^{2+}/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 $[Ca^{2+}]_i$ measurements, cells were used 48–72 h after plating.

Fura-2 Imaging of Intracellular Ca^{2+}—Cytosolic 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 $CaCl_2$ and 1 mM $MgCl_2$ in the presence of 5 μM Fura-2/AM and 1 mg/ml Pluronic F-127 dissolved in Me_2SO 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 ~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: $[Ca^{2+}]_i = K_d \times ((R - R_{min})/(R_{max} - R)) \times (S_{f380}/S_{b380})$ 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_{f380} and S_{b380} 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 μM) 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 (isosbestic wavelength) in the presence of $MnCl_2$ (500 μM) 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 μg/ml aprotinin (Sigma), 100 μg/ml leupeptin (Sigma), and 100 μg/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 $P2X_4$ (Alomone Laboratories, Jerusalem, Israel) at a dilution of 1:500. $P2X_1$, $P2X_2$, and $P2X_7$ 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^2. 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 μl of a stock solution containing 30 mM $NaIO_4$ and 600 μl 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

Δratios (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 μM), except ADP (100 μM) +Ca^{2+} free media whose value for % is *versus* ADP (100 μM). Values are means ± S.D.; n = number of experiments.

	Δratio	%	n
ATP (100 μM)	0.30 ± 0.11	100	15
ATP (10 μM)	0.20 ± 0.10	67	4
ATP (100 μM) + suramin (100 μM)	0.04 ± 0.02[a]	13	4
ATP (100 μM) + Ca^{2+}-free media	0.18 ± 0.03[a]	60	5
ADP (100 μM)	0.38 ± 0.17	127	8
ADP (100 μM) + Ca^{2+}-free media	0.16 ± 0.05[b]	42	4
ADPβS (100 μM)	0.37 ± 0.06	123	7
ADPβS (10 μM)	0.28 ± 0.07	93	3
2MeSATP (100 μM)	0.24 ± 0.06	80	3
ATP (100 μM) + $ZnCl_2$ (20 μM)	0.25 ± 0.07	83	4
ATP (100 μM) at pH_e = 7.9	0.38 ± 0.18	127	2
ATP (100 μM) at pH_e = 6.4	0.22 ± 0.02	73	2
UTP (100 μM)	No increase	0	6
UDP (100 μM)	No increase	0	3
Adenosine (100 μM)	No increase	0	4
BzBzATP (100 μM)	No increase	0	3
α,β-MeATP (100 μM)	No increase	0	3

[a] $p < 0.05$ relative to ATP (100 μM).
[b] $p < 0.05$ relative to ADP (100 μM).

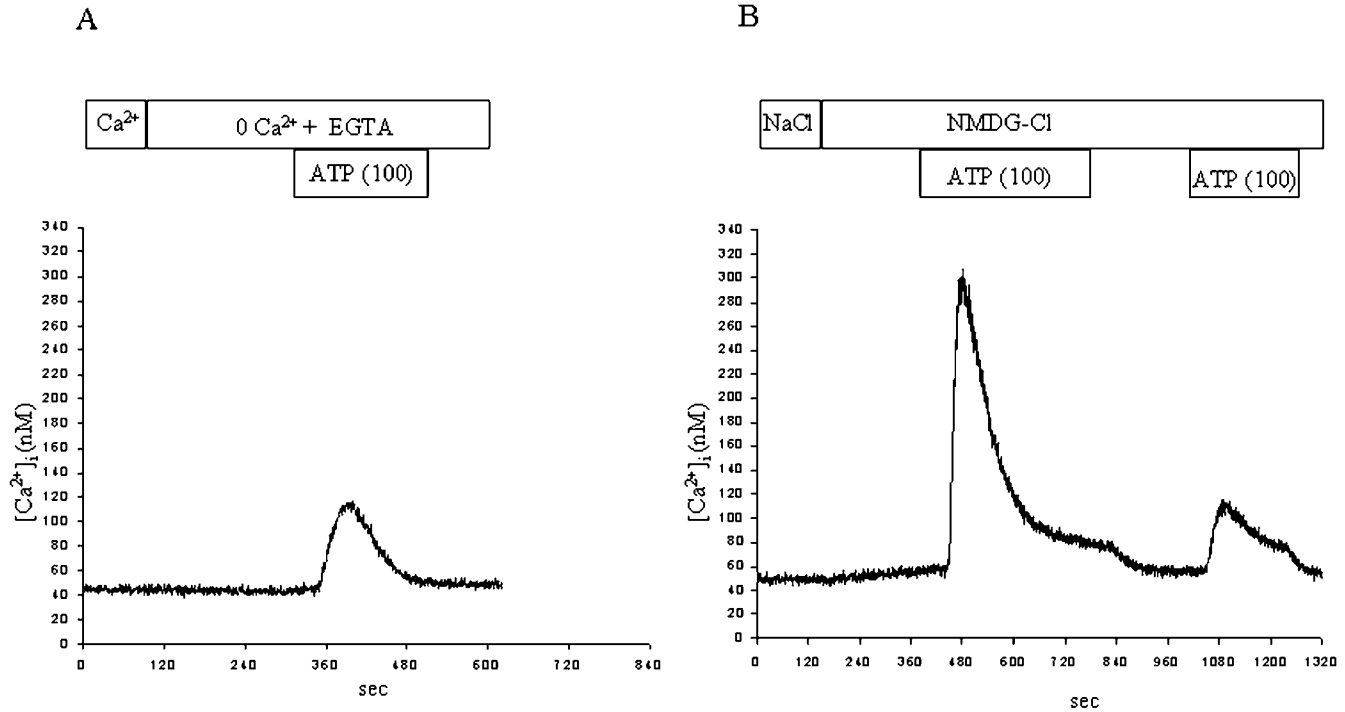

FIG. 2. **Original traces showing the effects of ATP (100 μM) on $[Ca^{2+}]_i$ in IB3-1 cells exposed to nominally Ca^{2+}-free, Na^+-containing solution (*A*) and in cells exposed to Ca^{2+}-containing Na^+-free solution (*B*) as indicated.** *B*, please note the slight sustained increase in $[Ca^{2+}]_i$ 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.

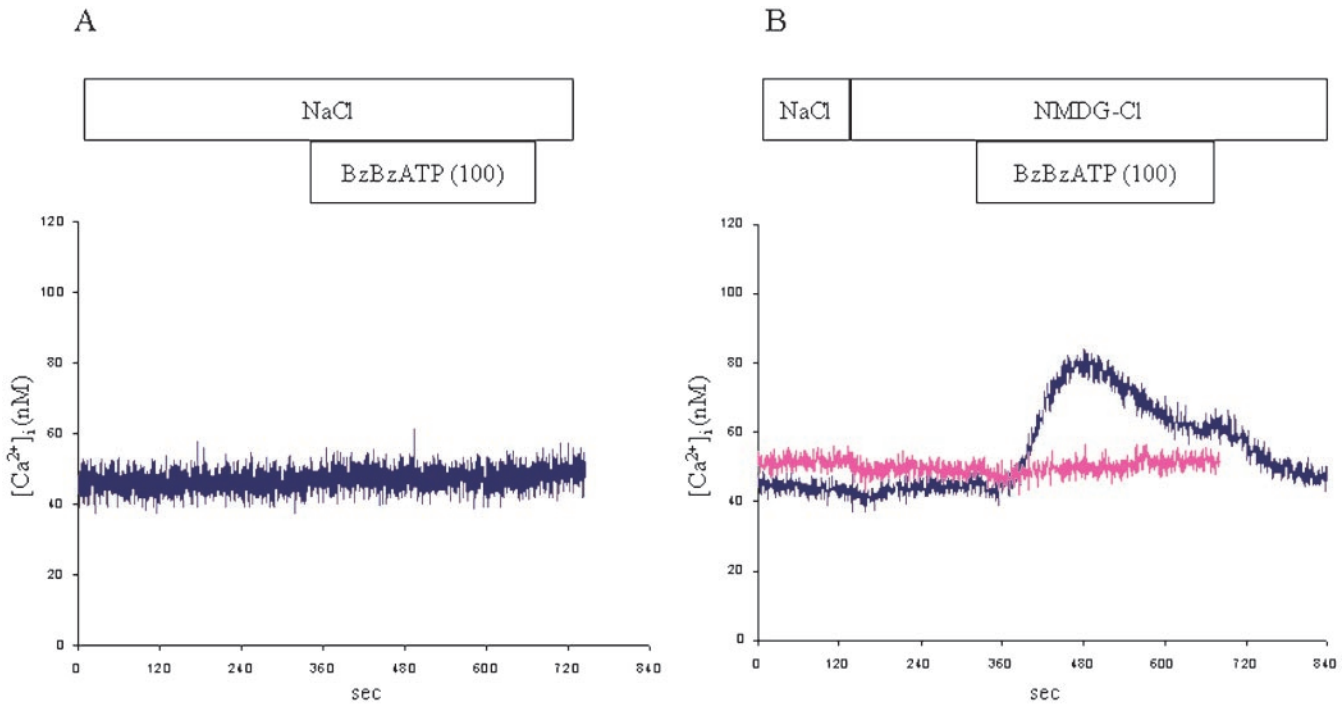

FIG. 3. *A* includes an original trace showing the lack of an effect of BzBzATP (100 μM) on $[Ca^{2+}]_i$ in IB3-1 cells in Na^+-containing medium. *B* shows an original trace where cells exposed to Na^+-free medium are responsive to BzBzATP (100 μM) with a rise in $[Ca^{2+}]_i$ in IB3-1 cells. Note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

Δratios (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 μM). Values are means ± S.D.; n = number of experiments.

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

[a] $p < 0.05$ relative to ATP (100 μM) in sodium-containing medium (see Table I).
[b] $p < 0.05$ relative to ATP (100 μM) alone.
[c] $p < 0.05$ relative to ADPβS (100 μM) in sodium-containing medium (see Table I).
[d] $p < 0.05$ relative to BzBzATP (100 μM) in sodium-containing medium (see Table I).

(Pierce) for 1 h at 4 °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 °C. Beads were washed 3 times with lysis buffer and incubated in sample buffer for 5 min at 95 °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 μM) was added to Ca^{2+}- and EGTA-free solutions.

Data Analysis—Data are expressed as mean ± 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 μM) caused a rapid increase in the ratio (340/380 nm) of Fura-2 fluorescence (r_{basal} = 0.89 ± 0.09 to r_{peak} = 1.19 ± 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 ± 0.09; n = 15) (Fig. 1*A*). 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. 1*A*). Administration of 10 μM 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 μM) (Table I). ADP, 2MeSATP (100 μM each), and ADPβS (10 and 100 μM) 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 ADPβS increased $[Ca^{2+}]_i$ in a similar manner to ATP and ADP, these data argue strongly for activation of $P2Y_1$ receptors over other P2Y subtypes. In contrast, neither UTP (100 μM) (Fig. 1*A*) nor UDP (100 μM) 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 μM).

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^+-containing medium. Values are means ± S.D.; n = number of experiments.

	Na^+-containing medium			Na^+-free medium		
	Δratio	%	n	Δratio	%	n
ATP (100 μM)	0.03 ± 0.02	100	15	0.10 ± 0.03[a]	333	11
ATP (100 μM) + Ca^{2+}-free media	No increase	0	5	No increase	0	3
ATP (100 μM) + $ZnCl_2$ (20 μM)	0.02 ± 0.01	66	4	0.26 ± 0.04[a,b]	866	5
ATP (100 μM) at pH_e = 7.9	0.04 ± 0.01	133	2	0.18 ± 0.02[a,b]	600	5
ATP (100 μM) at pH_e = 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[a]	466	3
ATP (100 μM) + high $[KCl]_e$ (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[a]	167	5
ATP (100 μM) + 2APB (75 μM)	0.02 ± 0.02	66	2	0.12 ± 0.04[a]	400	3
ATP (100 μM) + SKF-56365 (50 μM)	Not tested			0.25 ± 0.08[a,b]	833	4

[a] $p < 0.05$ relative to ATP (100 μM) in Na^+-containing medium.
[b] $p < 0.05$ relative to ATP (100 μM) in Na^+-free medium.

Because adenosine did not increase $[Ca^{2+}]_i$, we did not pursue the participation of P1 receptors in increasing $[Ca^{2+}]_i$ (Table I).

Purinergic Agonists Trigger a Transient Increase in $[Ca^{2+}]_i$ in the Absence of Extracellular Ca^{2+}—Activation of $P2Y_1$ receptors leads to G protein-coupled phospholipase C- and inositol 1,4,5-trisphosphate-dependent release of Ca^{2+} from intracellular stores. As such, P2Y agonists should increase cytosolic Ca^{2+} even in the absence of extracellular Ca^{2+}. Therefore, we repeated the experiments with ATP (100 μM) (Fig. 2*A*) and ADP (100 μM) superfusing IB3-1 cells with solutions containing EGTA (1 mM) instead of $CaCl_2$ (solution B). Similar to control conditions, both agonists increased $[Ca^{2+}]_i$ transiently, indicating that their effects, at least partially, were independent from extracellular Ca^{2+} (Table I). Nonetheless, the absence of extracellular Ca^{2+} reduced the agonist-induced peak increase in $[Ca^{2+}]_i$ (Table I). Again, under these conditions, the Ca^{2+} transients decayed fully back to base line within 200 s. Interestingly, these data did suggest that, besides $P2Y_1$ receptor activation, purinergic agonists may also trigger Ca^{2+} influx from extracellular stores, which contributes to the peak increase in $[Ca^{2+}]_i$. Nevertheless, under these ionic conditions, Ca^{2+} influx was not sufficient to support a sustained elevation of $[Ca^{2+}]_i$, the goal of this study. Experiments described below lend clarification to these early data.

P2X Receptor-selective Agonists Fail to Trigger an Increase in $[Ca^{2+}]_i$ in the Presence of Extracellular Na^+ and Ca^{2+}—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 $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} and the loss of the full response in Ca^{2+}-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. 3*A*), selective agonists for different P2X receptor subtypes. Under these conditions, P2X-selective purinergic agonists failed to change $[Ca^{2+}]_i$ (Table I). However, we were aware of the fact that α,β-MeATP and BzBzATP, although potent agonists at $P2X_1$, $P2X_3$, and $P2X_7$ 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^{2+} entry mechanism by these agonists (see below).

ATP and BzBzATP Trigger an Increase in $[Ca^{2+}]_i$ with Transient and Sustained Components in the Absence of Extracellular Na^+—Despite the negative data above with regard to P2X-selective agonists, we maintained the hypothesis that P2X receptors were involved in the full Ca^{2+} response induced by ATP in the presence of extracellular Ca^{2+}. Rationale for this hypothesis is given by the fact that, in human and mouse lymphocytes, Na^+ might compete with Ca^{2+} for entry through P2X receptors from extracellular stores (36–38) as well as other families of Ca^{2+} entry channels like the transient receptor potential channels (TRPs) or the store-operated Ca^{2+} channels (SOCs) (39, 40). Thus, we speculated that extracellular Na^+ might suppress the Ca^{2+} permeability of P2X receptor channels in IB3-1 cells. To verify this hypothesis, we substituted extracellular Na^+ 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 $P2Y_1$-specific agonist (ADPβS). As shown in Fig. 2*B* (and in Fig. 5*B* and Fig. 7, *A* and *B*), substitution of extracellular Na^+ by NMDG itself caused a small but sustained increase in $[Ca^{2+}]_i$ ($r_{basal} = 0.89 \pm 0.04$ to $r_{NMDG} = 0.94 \pm 0.04$; $n = 31$; $p < 0.05$) which was completely absent when extracellular Ca^{2+} was also omitted from the superfusion medium. These observations suggest the presence of a mechanism that allows sustained Ca^{2+} entry, even in non-stimulated cells.

Following removal of extracellular Na^+ and changes in $[Ca^{2+}]_i$, we applied ATP (100 μM). Under these conditions, ATP induced a further increase in $[Ca^{2+}]_i$ displaying a biphasic Ca^{2+} response consisting of an initial transient peak and a sustained component (Fig. 2*B* and Tables II and III). In addition, as shown in Fig. 2*B*, a second application of ATP elicited a smaller increase in the $[Ca^{2+}]_i$ peak; however, the sustained Ca^{2+} plateau was comparable with that observed after the first stimulation by ATP. When $[Ca^{2+}]_i$ reached a stable value after withdrawal of extracellular Na^+, we also added either BzBzATP (100 μM) (Fig. 3*B*) or α,β-MeATP (100 μM). BzBzATP, but not α,β-MeATP, induced a small increase in $[Ca^{2+}]_i$ (Fig. 3*B* and Tables II and III). This increase was completely dependent on the presence of extracellular Ca^{2+}, indicating a role for P2X receptors in Ca^{2+} influx (Fig. 3*B* and Table II). In Na^+-free media, $P2Y_1$-specific agonist, ADPβS (100 μM), augmented the peak increase in $[Ca^{2+}]_i$ (Table II) but failed to elicit a sustained Ca^{2+} plateau (Table III). Taken together, these data argue for a role for P2X receptors as Ca^{2+} entry channels in IB3-1 cells.

$P2X_4$ 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 $P2X_4$ receptor channel robustly. Membrane protein lysates from IB3-1 cells were prepared and were subjected to immunoblotting with a $P2X_4$-specific polyclonal antibody. Fig. 4*A* shows the positive results for $P2X_4$ 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 $P2X_1$, $P2X_2$, and $P2X_7$ using specific antibodies to those subtypes (data not shown). The $P2X_4$ 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 $P2X_4$ receptor biochemistry in cardiac tissue and myocytes (42). An unglycosylated band was detected at ~46 kDa (the predicted molecular mass for $P2X_4$) and a larger and broader glycosylated band at 60–65 kDa. These immunoblotting data show that $P2X_4$ 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 $P2X_4$ receptor channels as the major P2X receptor subtype mediating Ca^{2+} entry (see below).

Fig. 4, *B–D,* shows additional data in 16HBE14o$^-$ non-CF airway epithelial cells. Immunoblotting of non-polarized cells grown in flasks (Fig. 4, *B* and *C*) as well as biotinylation (Fig. 4*D*) of polarized monolayers grown on permeable supports revealed robust and apical membrane-localized expression of $P2X_4$. 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. 4*D*, 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 $P2X_4$ receptor expression (data not shown). These data suggest that $P2X_4$ receptors are expressed abundantly by human airway epithelial cells grown under non-polarized and polarized conditions.

The Extracellular ATP-gated $P2X_4$ Receptor Channel Is the Major Ca^{2+} Entry Channel Stimulated by ATP in IB3-1 and 16HBE14o$^-$ Cells—Like other subtypes of the P2X receptor channel family, the $P2X_4$ receptors are also regulated by different cations, such as H^+ or Zn^{2+} (25). Thus, if it is true that in IB3-1 cells the prolonged Ca^{2+} response in Na^+-free medium was due to activation of $P2X_4$ receptors, then extracellular pH and Zn^{2+} should modify the ATP-induced Ca^{2+} signal. To test this hypothesis, we measured $[Ca^{2+}]_i$ after changing extracellular pH or in the presence of Zn^{2+} in both IB3-1 and 16HBE14o$^-$ 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 $[Ca^{2+}]_i$ only in Na^+-free medium. Furthermore, in a Na^+-free environment, acidic pH significantly reduced the ATP-induced peak increase in $[Ca^{2+}]_i$ (Table II). To demonstrate directly the effect of ATP on Ca^{2+} influx from extracellular sources via another approach, we measured quenching of Fura-2 at 359 nm in the presence of $MnCl_2$ (500 μM). Mn^{2+} is known to permeate the same entry channels as Ca^{2+} and quenches Fura-2 fluorescence when it enters the cells. As shown in Fig. 5*A*, in Na^+-free medium, acidic extracellular pH (6.4) inhibited Mn^{2+} entry, whereas alkaline extracellular pH (7.9) potentiated markedly Mn^{2+} entry and quenching of the dye. To further support the involvement of $P2X_4$ receptor channels, we tested the effect of the P2X receptor co-agonist, Zn^{2+}, on ATP-induced Ca^{2+} entry mechanisms. Inclusion of $ZnCl_2$ (20 μM) further augmented the sustained increase in $[Ca^{2+}]_i$ induced by ATP in Na^+-free medium (Fig. 5*B* and Table III) but had no effect in Na^+-containing medium

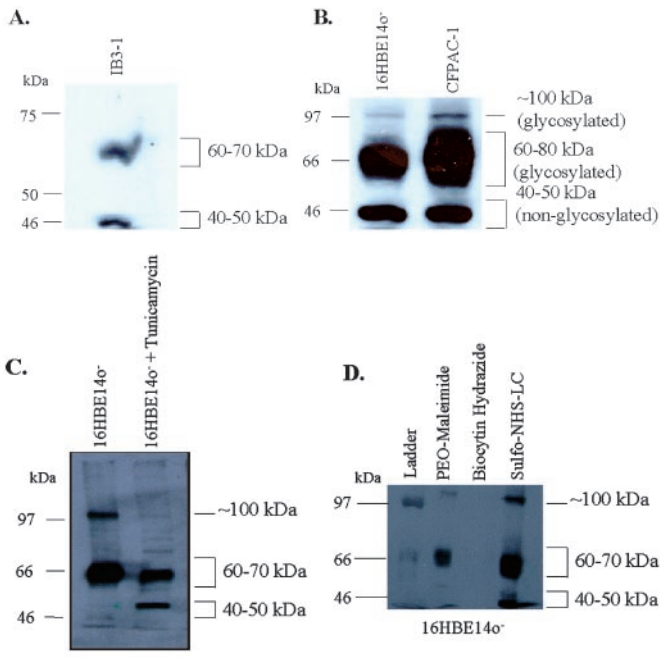

FIG. 4. *A*, immunoblot analysis of IB3-1 cells grown as non-polarized monolayers in flasks using rabbit polyclonal antibodies against $P2X_4$ receptors. A smaller band of the predicted molecular mass for $P2X_4$ (46 kDa) was detected, as was a larger, broader, glycosylated band at 60–70 kDa. The positions of molecular mass markers are shown on the *left* (in kDa). This is representative of 3 such experiments. *B*, immunoblot analysis of 16HBE14o$^-$ 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 16HBE14o$^-$ 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 $P2X_4$. The data reveal that poly(ethylene)oxid-maleimide biotin, a reagent that reagents with primary amines primarily on lysine residues, detected only the glycosylated forms in the apical plasma membrane of 16HBE14o$^-$ 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 $P2X_4$; 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 $P2X_1$, $P2X_2$, and $P2X_7$ did not block the $P2X_4$ 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 $P2X_4$ receptor protein.

(Table III). Since our biochemical data (see above) indicated that $P2X_4$ receptors are also present in 16HBE14o$^-$ non-CF airway epithelial cells, we tested whether increasing extracellular pH or addition of Zn^{2+} augmented the ATP-induced sustained Ca^{2+} entry in Na^+-free medium in 16HBE14o$^-$ cells. As shown in Fig. 6*A*, ATP elicited extracellular pH-dependent quenching of Fura-2, suggesting that ATP-stimulated Ca^{2+} influx is facilitated by alkaline pH. In addition, similar to results obtained with IB3-1 cells, both inclusion of Zn^{2+} and increasing pH potentiated the effects of ATP on sustained Ca^{2+} signal (Fig. 6*B*). Taken together, these data argue for a prom-

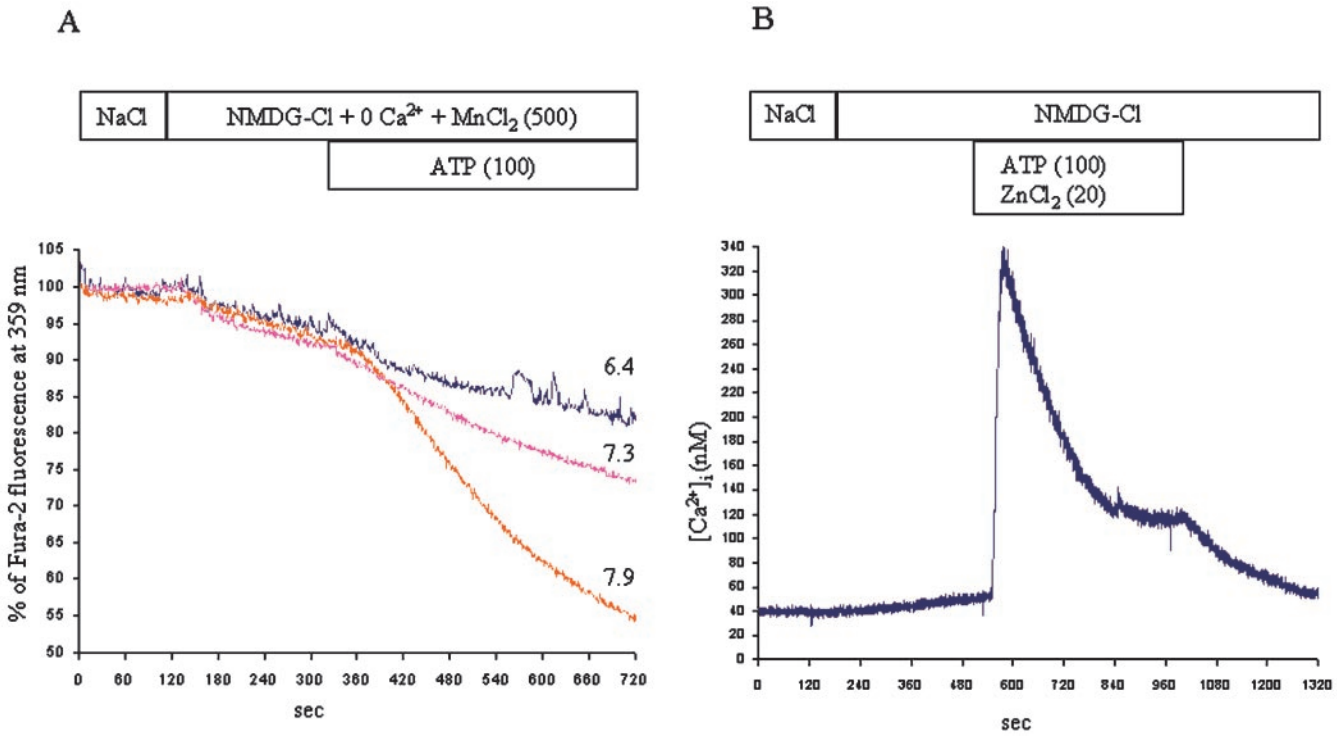

FIG. 5. **Representative traces showing the pH dependence of ATP-induced Mn^{2+} entry in IB3-1 cells.** *A*, quenching of Fura-2 was measured at the isosbestic wavelength of Fura-2 (359 nm). Cells were exposed to $MnCl_2$ (500 μM) in Na^+- and Ca^{2+}-free medium at pH 7.3. After 200 s, ATP (100 μM) 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 μM) in presence of $ZnCl_2$ (20 μM) 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. 2*B*).

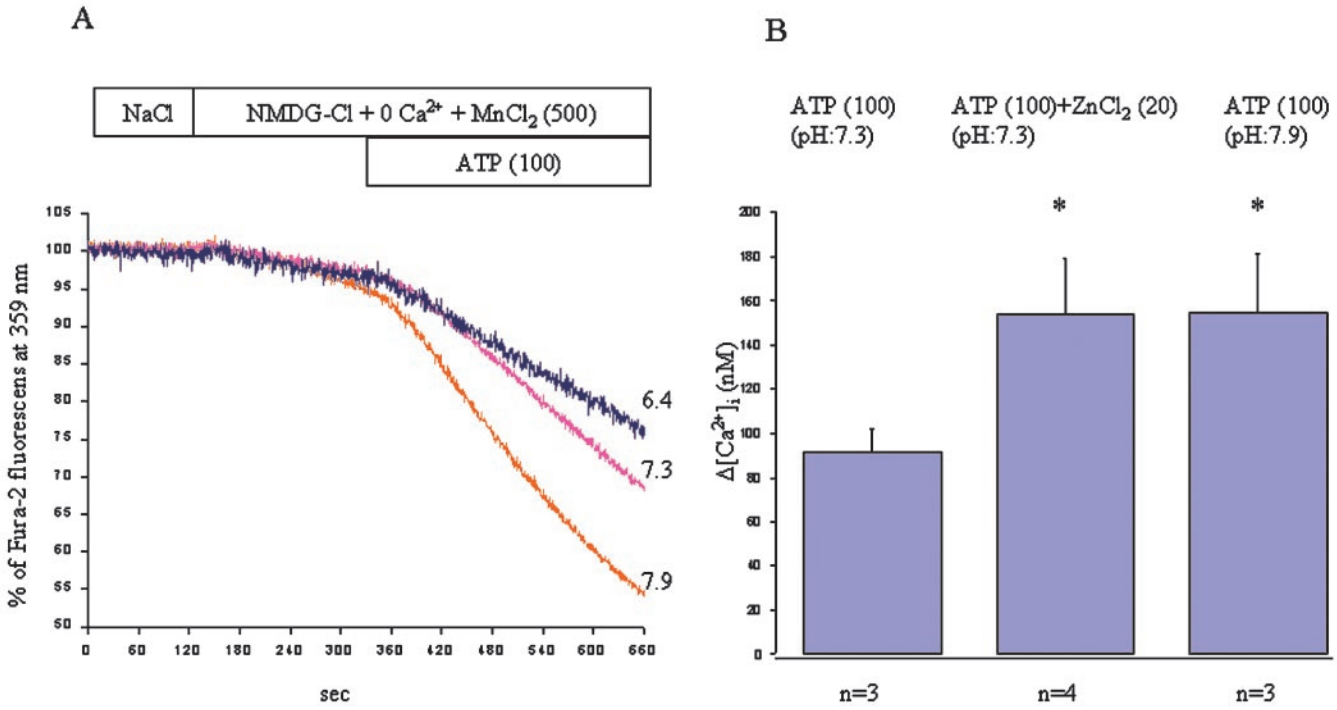

FIG. 6. **Representative traces on the *left* showing the pH dependence of ATP-induced Mn^{2+} entry in 16HBE14o$^-$ cells.** *A*, experiments were performed in a similar manner to those in Fig. 5*A*. Changes in cytosolic Ca^{2+} concentration 5 min after the peak stimulation *versus* the basal $[Ca^{2+}]_i$ 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

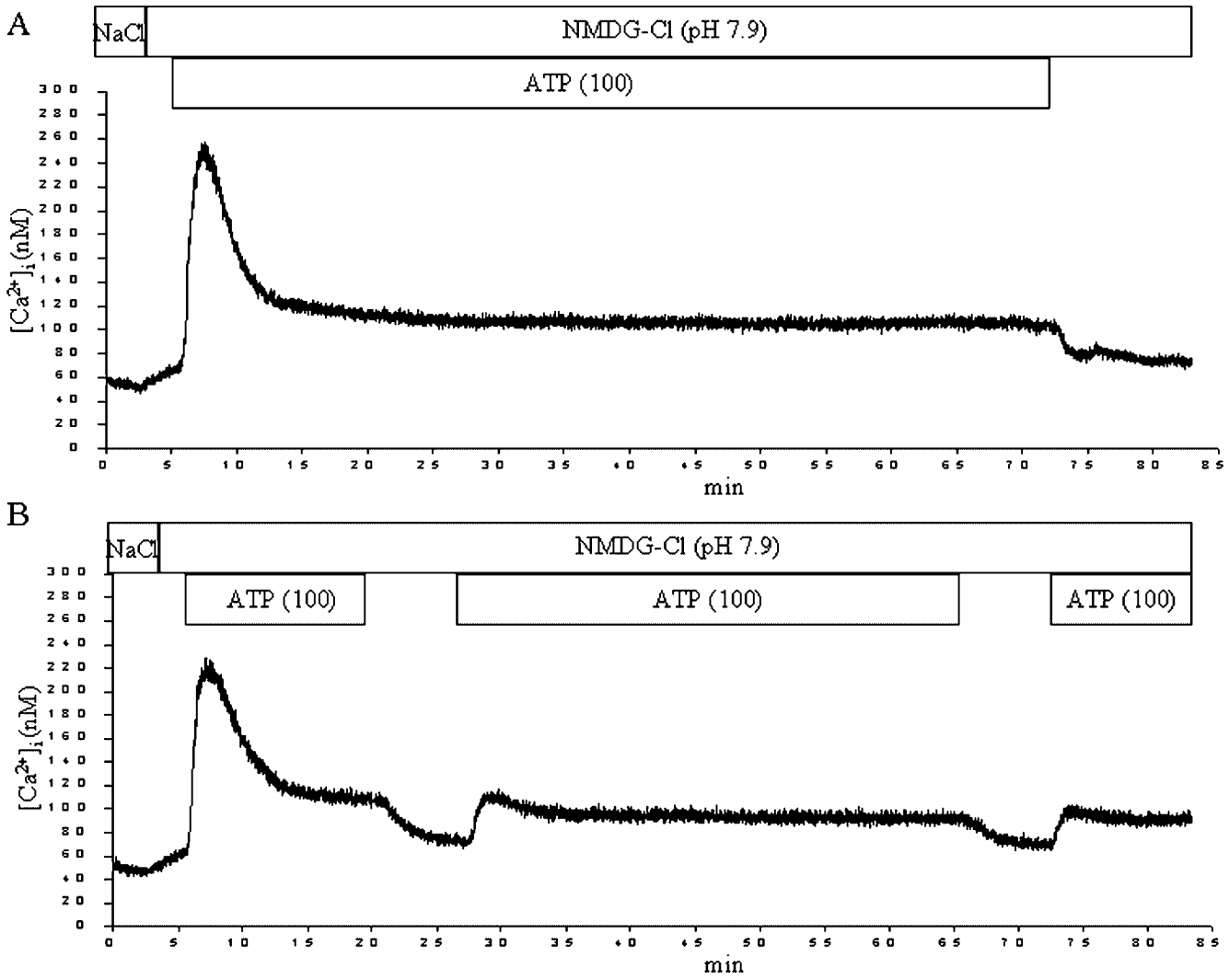

FIG. 7. **Representative traces showing the duration of the sustained plateau in $[Ca^{2+}]_i$ in IB3-1 CF cells induced by ATP under Na^+-free conditions (pH 7.9) (*A*) and the reversibility, long lived, and reproducible nature of the sustained plateau induced by ATP and mediated by $P2X_4$ (*B*).** 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 μM) 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. 7*A*). 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. 7*B*), 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 μM), 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. 8*A* 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. 8*B* 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 SOCs and TRPs could be responsible for the sustained Ca^{2+} influx induced by ATP in Na^+-free

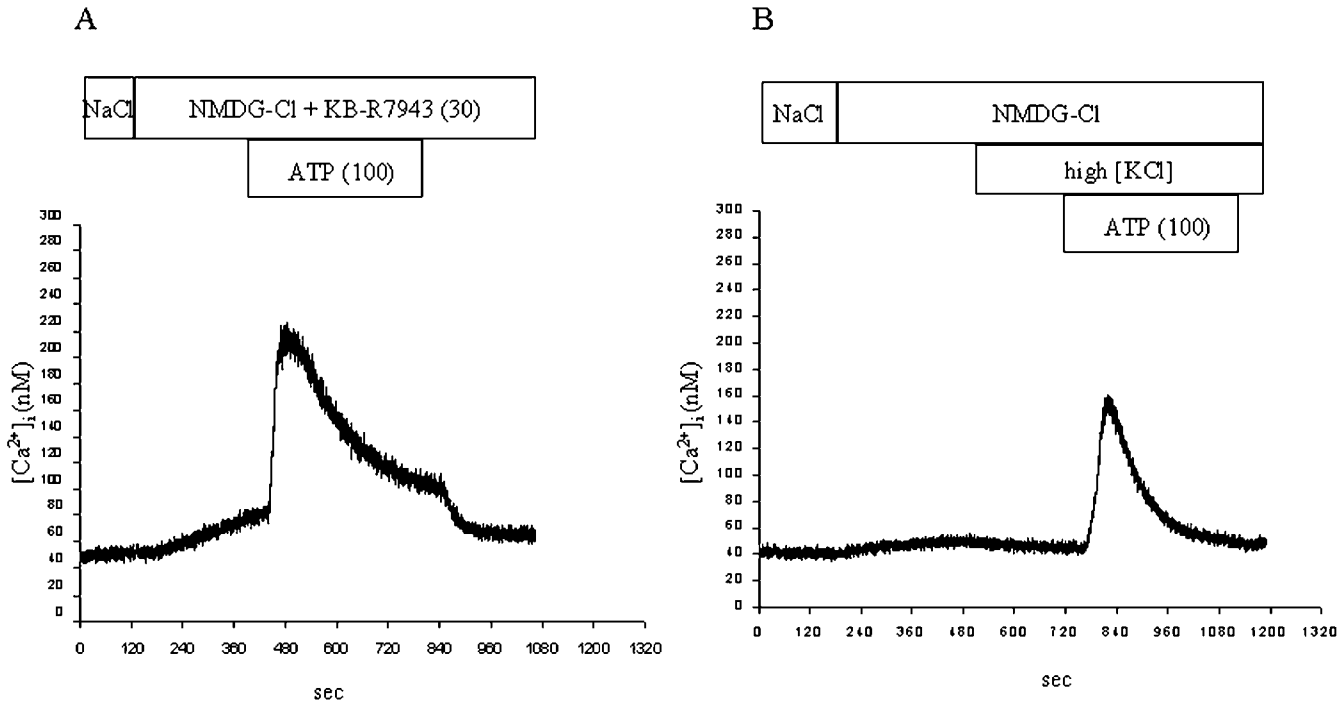

FIG. 8. **Representative traces showing the effects of KB-R7943 (30 μM) (*A*) and high $[KCl]_e$ (40 mM) (*B*) on ATP-induced Ca^{2+} signal.** Experiments were done in a Na^+-free environment. *B*, note that substitution of Na^+ by NMDG causes a slight increase in $[Ca^{2+}]_i$, an effect that was inhibited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

medium. Therefore, we tested whether SOCs 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_{basal} = 1.00 ± 0.05 to r_{peak} = 2.92 ± 0.17; n = 3) followed by a sustained Ca^{2+} plateau ($r_{sustained}$ = 1.58 ± 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 SOCs, which are activated by a decrease in $[Ca^{2+}]_{ER}$. Next, we have asked whether SOCs 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 SOCs (44, 45), and SKF-96365, which is a blocker of the store-independent TRPs (46). Neither 2APB (75 μM) nor SKF-56365 (50 μM) 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, SOCs and/or TRPs do not play a role in regulating $[Ca^{2+}]_i$ following purinergic receptor stimulation.

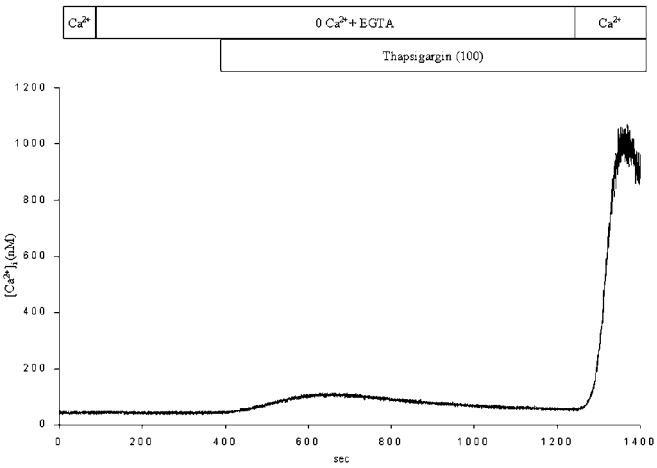

FIG. 9. **Representative trace shows the effect of thapsigargin (100 nM) on $[Ca^{2+}]_i$ in the absence and in the presence of extracellular Ca^{2+} as indicated.** This maneuver reveals the presence of SOCs involved in Ca^{2+} entry, albeit induced by emptying ER stores completely.

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). ADPβS, 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, ADPβS at a concentration of 100 μM may 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, ADPβS also elicited a significant increase in $[Ca^{2+}]_i$ at a concentration of 10 μM. 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.[2] 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 $P2X_4$.

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 $P2X_4$ 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 $P2Y_1$ receptor-specific agonist, ADPβS, 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 $P2X_4$. 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 $P2X_7$ 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 $P2X_4$ receptors and inconsistent with other P2X receptor subtypes (25). For instance, stimulatory effects by Zn^{2+} rule out a role for $P2X_7$, because Zn^{2+} is a $P2X_7$ antagonist (25). Inhibition of Ca^{2+} entry by acidic pH rules out $P2X_2$ receptors, which are stimulated by acidic pH (25). The only phenotype that is not completely explained by $P2X_4$ alone is the alkaline pH stimulation. Heterologously expressed $P2X_4$ is only mildly stimulated by alkaline pH (64). As such, we cannot rule out that additional P2X receptor subtypes (perhaps $P2X_5$ (65), $P2X_6$ (66), or splice variants of $P2X_4$, $P2X_5$, and $P2X_6$ (67)) may be conferring these pH effects in a P2XR heteromultimer. Interestingly, in 16HBE14o$^-$ cells, ATP-driven Mn^{2+} entry was also enhanced by alkaline pH, and Zn^{2+} potentiated the ATP-induced sustained increase in $[Ca^{2+}]_i$. Taken together, these data indicate that $P2X_4$ 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 16HBE14o$^-$ non-CF cells, extracellular Na^+ (140 mM) prevented neither the BzBzATP-dependent Ca^{2+} response nor the ATP-induced Ca^{2+} plateau[3]; 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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