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

Received for publication, December 3, 2002, and in revised form, January 21, 2003
Published, JBC Papers in Press, February 3, 2003, DOI 10.1074/jbc.M212277200

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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\(_{\text{2+}}\)]\(_i\), in human CF (IB3-1) and non-CF (16HBE14o-) airway epithelial cells. Cytosolic Ca\(_{\text{2+}}\) was measured by fluorospectrometry using the fluorescent dye Fura-2/AM. Expression of P2X receptor (P2XR) subtypes was assessed by immunoblotting and biontinylaton. In IB3-1 cells, ATP and other P2Y agonists caused only a transient increase in [Ca\(_{\text{2+}}\)]\(_i\), derived from intracellular stores in a Na\(^+\)-rich environment. In contrast, ATP induced an increase in [Ca\(_{\text{2+}}\)]\(_i\), 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\(_{\text{2+}}\)]\(_i\), only in Na\(^+\)-free medium, suggesting competition between Na\(^+\) and Ca\(_{\text{2+}}\) through P2XRs. Biochemical evidence showed that the P2Xr receptor is the major subtype shared by these airway epithelial cells. A role for store-operated Ca\(_{\text{2+}}\) channels, voltage-dependent Ca\(_{\text{2+}}\) channels, or Na\(^+\)/Ca\(_{\text{2+}}\) exchanger in the ATP-induced sustained Ca\(_{\text{2+}}\) signal was ruled out. In conclusion, these data show that epithelial P2Xr receptors serve as ATP-gated calcium entry channels that induce a sustained increase in [Ca\(_{\text{2+}}\)]\(_i\). In airway epithelia, a P2XR-mediated Ca\(_{\text{2+}}\) signal may have therapeutic benefit for CF.

In cystic fibrosis (CF), 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\(_{\text{2+}}\)-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\(_{\text{2+}}\) concentration ([Ca\(_{\text{2+}}\)]\(_i\)) 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-hydroryzable UTP analogs as therapeutic agonists targeted to the P2Y\(_t\) 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\(_{\text{2+}}\), 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\(_{\text{2+}}\)]\(_i\), 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\(_{\text{2+}}\) 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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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 (DELRe-508) and a premature stop codon mutation (W1282X). IB3-1 cells were cultured in LHC-8 (Biofluids, Rockville, MD) medium supplemented with 5% fetal bovine serum (Invitrogen), 100 units/ml penicillin/streptomycin, and 1.5 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 Ca2+-free PBS. The cells were then suspended using trypsin/EDTA solution and plated on diluted Vitrogen-coated (collagen types I and IV) glass coverslips. For [Ca2+]i measurements, cells were used 48–72 h after plating.

Fura-2 Imaging of Intracellular Ca2+—Cytosolic Ca2+ 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 CaCl2 and 1 mM MgCl2 in the presence of 5 μM Fura-2/AM and 1 mg/ml Pluronic F-127 dissolved in Me2SO for 120 min to allow loading of the dye into the cells. After loading, coverslips were rinsed at least for 10 min in phosphate-buffered saline to remove extracellular Fura-2/DMSO. The cells were then 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.

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 [Ca2+]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.

Epithelial P2X receptors function as ATP-gated Ca2+ subtype present in both cell lines. Thus, we conclude that P2Y receptors elicit a transient increase in [Ca2+]i (30). IB3-1 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% CO2 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 Ca2+-/Mg2+-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 [Ca2+]i measurements, cells were used 48–72 h after plating.

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~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 then added to the appropriate solutions (see later). The 340/380 nm ratios (R) were then added to the appropriate solutions (see later).

**TABLE I**

| 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 | 13 4 |
| ATP (100 μM) + Ca²⁺-free media | 0.18 ± 0.03 | 60 5 |
| ADP (100 μM) | 0.38 ± 0.17 | 127 8 |
| ADP (100 μM) + Ca²⁺-free media | 0.16 ± 0.05 | 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) + ZnCl₂ (20 μM) | 0.25 ± 0.07 | 83 4 |
| ATP (100 μM) at pH 7.9 | 0.38 ± 0.18 | 127 2 |
| ATP (100 μM) at pH 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 |
| αβ,β-MeATP (100 μM) | No increase | 0 3 |
| αβ,β-MeATP (100 μM) | No increase | 0 3 |

*p < 0.05 relative to ATP (100 μM).

**Fura-2 Quenching Experiments**—Cells were loaded and washed as described for intracellular [Ca²⁺] measurement. Fluorescence signal was measured at 359 nm (isosbestic wavelength) in the presence of MnCl₂ (500 μM) to detect Ca²⁺-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 polyvinylidine difluoride membrane (Osmonics, Westboro, MA). Immunoblotting was performed with a rabbit polyclonal antibody to P2X₄ (Alomone Laboratories, Jerusalem, Israel) at a dilution of 1:500. P2X₁, P2X₂, and P2X₃ 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/cm². Cells were placed on ice and washed 3 times with cold PBS supplemented with 0.1 mM CaCl₂ and 1.0 mM MgCl₂. Cells were then incubated in 1.0 mg/mlpoly(ethylene)oxid maleimide (Pierce) or sulfo-NHS-LC biotin (Pierce) in cold supplemented PBS for 30 min at room temperature in the dark. Filters 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 NaIO₄ 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

![Fig. 2. Original traces showing the effects of ATP (100 μM) on [Ca²⁺], in IB3-1 cells exposed to nominally Ca²⁺-free, Na⁺-containing solution (A) and in cells exposed to Ca²⁺-containing Na⁺-free solution (B) as indicated. B, please note the slight sustained increase in [Ca²⁺], upon substitution of Na⁺ by NMDG. This sustained plateau was the first hint that in Na⁺-free medium Ca²⁺ entry channels could also be involved in the ATP-induced sustained Ca²⁺ response.](http://www.jbc.org/)
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Purinergic Agonists Trigger a Transient Increase in [Ca\textsuperscript{2+}], in the Presence of Extracellular Na\textsuperscript{+} in IB3-1 Cells—To test for the presence of purinergic receptors in IB3-1 cells, we measured the cytosolic free Ca\textsuperscript{2+} concentration after stimulation with different agonists to both P2Y and P2X receptors in physiologic bath solution (solution A) containing Na\textsuperscript{+}. Superfusion of cells with solution containing ATP (100 \mu M) caused a rapid increase in the ratio (340/380 nm) of Fura-2 fluorescence \((r_{basal} = 0.89 \pm 0.09\) to \(r_{peak} = 1.19 \pm 0.13\); \(n = 15\)). However, the response was transient, and the [Ca\textsuperscript{2+}] 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\textsuperscript{2+}] (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\textsuperscript{S} (10 and 100 \mu M) also caused an increase in cytosolic Ca\textsuperscript{2+} concentration, showing similar characteristics described for ATP (Fig. 1, B–D, and Table I). Because 2MeSATP and ADP\textsuperscript{S} increased [Ca\textsuperscript{2+}], in a similar manner to ATP and ADP, these data argue strongly for activation of P2Y\textsubscript{1} receptors over other P2Y subtypes. In contrast, neither UTP (100 \mu M) (Fig. 1A) nor UDP (100 \mu M) had any effect on Ca\textsuperscript{2+} concentration (Table I). To explore whether degradation of ATP or ADP plays role in elevation of [Ca\textsuperscript{2+}], we tested the effects of adenosine (100 \mu M).

# RESULTS

**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.

**TABLE II**

| \(\Delta r\) 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 ± S.D.; \(n = \) number of experiments. | \(\Delta r\) ratio | % | \(n\) |
|---|---|---|---|
| ATP (100 \mu M) | 0.82 ± 0.24 | 100 | 11 |
| ATP (100 \mu M) + Ca\textsuperscript{2+}-free media | 0.25 ± 0.14 | 31 | 4 |
| ATP (100 \mu M) + ZnCl\textsubscript{2} (20 \mu M) | 0.77 ± 0.21 | 94 | 5 |
| ATP (100 \mu M) at \(pH_{i} = 7.9\) | 0.78 ± 0.22 | 95 | 5 |
| ATP (100 \mu M) at \(pH_{i} = 6.4\) | 0.22 ± 0.06 | 27 | 5 |
| ATP (100 \mu M) + KB-R7943 (30 \mu M) | 0.70 ± 0.13 | 85 | 3 |
| ATP (100 \mu M) + high [KCl] (40 mM) | 0.13 ± 0.06 | 66 | 4 |
| ADP\textsuperscript{S} (100 \mu M) | 0.80 ± 0.02 | 95 | 3 |
| BzBzATP (100 \mu M) | 0.15 ± 0.02 | 11 | 5 |
| BzBzATP (100 \mu M) + Ca\textsuperscript{2+}-free media | No increase | 0 | 3 |
| \(\alpha,\beta\text{-MeATP (100 \mu M)}\) | No increase | 0 | 3 |

\(*p < 0.05\) relative to ATP (100 \mu M) in sodium-containing medium (see Table I).

\(*p < 0.05\) relative to ATP (100 \mu M) alone.

\(*p < 0.05\) relative to ADP\textsuperscript{S} (100 \mu M) in sodium-containing medium (see Table I).

\(*p < 0.05\) relative to BzBzATP (100 \mu M) 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\textsuperscript{2+}], measurement contained (mmol/liter) the following: for solution A: NaCl 140, KCl 3, KH\textsubscript{2}PO\textsubscript{4} 1.3, Na\textsubscript{2}HPO\textsubscript{4} 8, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 2; for solution B: NaCl 140, KCl 3, KH\textsubscript{2}PO\textsubscript{4} 1.3, Na\textsubscript{2}HPO\textsubscript{4} 8, MgCl\textsubscript{2} 1, Na-EGTA 1; for solution C: NMDG-Cl 140, KCl 4.5, Hepes 10, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 2; and for solution D: NMDG-Cl 100, KCl 40, Hepes 10, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 2. The solutions are at pH 7.3 unless indicated otherwise. In Fura-2 quenching experiments MnCl\textsubscript{2} (500 \mu M) was added to Ca\textsuperscript{2+}- and EGTA-free solutions.
Because adenosine did not increase \([\text{Ca}^{2+}]_{i}\), we did not pursue the participation of P1 receptors in increasing \([\text{Ca}^{2+}]_{i}\) (Table I).

**Purinergic Agonists Trigger a Transient Increase in \([\text{Ca}^{2+}]_{i}\) in the Absence of Extracellular \([\text{Ca}^{2+}]_{i}\)**—Activation of P2Y receptors leads to G protein-coupled phospholipase C- and inositol 1,4,5-trisphosphate-dependent release of \([\text{Ca}^{2+}]_{i}\) from intracellular stores. As such, P2Y agonists should increase cytosolic \([\text{Ca}^{2+}]_{i}\) even in the absence of extracellular \([\text{Ca}^{2+}]_{i}\). Therefore, we repeated the experiments with ATP (100 \text{ M}) (Fig. 2A) and ADP (100 \text{ M}) superfusing IB3-1 cells with solutions containing EGTA (1 \text{ mM}) instead of \text{CaCl}_2 solution B. Similar to control conditions, both agonists increased \([\text{Ca}^{2+}]_{i}\), transiently, indicating that their effects, at least partially, were independent from extracellular \([\text{Ca}^{2+}]_{i}\) (Table I). Nonetheless, the absence of extracellular \([\text{Ca}^{2+}]_{o}\) reduced the agonist-induced peak increase in \([\text{Ca}^{2+}]_{i}\) (Table I). Again, under these conditions, the \([\text{Ca}^{2+}]_{i}\) transients decayed fully back to base line within 200 s. Interestingly, these data did suggest that, besides P2Y receptor activation, purinergic agonists may also trigger \([\text{Ca}^{2+}]_{i}\) influx from extracellular stores, which contributes to the peak increase in \([\text{Ca}^{2+}]_{i}\). Nevertheless, under these ionic conditions, \([\text{Ca}^{2+}]_{i}\) influx was not sufficient to support a sustained elevation of \([\text{Ca}^{2+}]_{i}\), the goal of this study. Experiments described below lend clarification to these early data.

**P2X Receptor-selective Agonists Fail to Trigger an Increase in \([\text{Ca}^{2+}]_{i}\) in the Presence of Extracellular \([\text{Na}^{+}]_{i}\) and \([\text{Ca}^{2+}]_{i}\)**—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 \([\text{Ca}^{2+}]_{i}\), in the presence of extracellular \([\text{Ca}^{2+}]_{i}\) and the full loss of the response in \([\text{Ca}^{2+}]_{i}\)-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 \(\alpha,\beta\)-methylene ATP (\(\alpha,\beta\)-MeATP, 100 \text{ M}) or benzoyl-benzoyl-ATP (BzBzATP, 100 \text{ M}) (Fig. 3A), selective agonists for different P2X receptor subtypes. Under these conditions, P2X-selective purinergic agonists failed to change \([\text{Ca}^{2+}]_{i}\) (Table I). However, we were aware of the fact that \(\alpha,\beta\)-MeATP and BzBzATP, although potent agonists at P2X_1, P2X_2, and P2X_3 receptors, have little or no effect at other P2X receptor subtypes. Thus, we hypothesized that changing the ionic composition of the superfusion medium might reveal activation of a \([\text{Ca}^{2+}]_{i}\) entry mechanism by these agonists (see below).

**ATP and BzBzATP Trigger an Increase in \([\text{Ca}^{2+}]_{i}\) with Transient and Sustained Components in the Absence of Extracellular \([\text{Na}^{+}]_{i}\)**—Despite the negative data above with regard to P2X-selective agonists, we maintained the hypothesis that P2X receptors were involved in the full \([\text{Ca}^{2+}]_{i}\) response induced by ATP in the presence of extracellular \([\text{Ca}^{2+}]_{i}\). Rationale for this hypothesis is given by the fact that, in human and mouse lymphocytes, \([\text{Na}^{+}]_{i}\) might compete with \([\text{Ca}^{2+}]_{i}\) for entry through P2X receptors from extracellular stores (36–38) as well as other families of \([\text{Ca}^{2+}]_{i}\)-entry channels such as the transient receptor potential channels (TRPs) or the store-operated \([\text{Ca}^{2+}]_{i}\)-channels (SOCs) (39, 40). Thus, we speculated that extracellular \([\text{Na}^{+}]_{i}\) might suppress the \([\text{Ca}^{2+}]_{i}\) permeability of P2X receptor channels in IB3-1 cells. To verify this hypothesis, we substituted extracellular \([\text{Na}^{+}]_{i}\) 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 \(\alpha,\beta\)-MeATP), and a P2Y-specific agonist (ADPβS). As shown in Fig. 2B (and in Fig. 5B and Fig. 7, A and B), substitution of extracellular \([\text{Na}^{+}]_{i}\) by NMDG itself caused a small but sustained increase in \([\text{Ca}^{2+}]_{i}\) (Table II) but failed to elicit 

**Table III**

| Na⁺-containing medium | Δratio | % | n | Na⁺-free medium | Δratio | % | n |
|------------------------|--------|---|---|-----------------|--------|---|---|
| ATP (100 μM)           | 0.03 ± 0.02 | 100 | 15 | 0.10 ± 0.03a | 333 | 11 |
| ATP (100 μM) + Ca²⁺-free media | No increase | 0 | 5 | No increase | 0 | 3 |
| ATP (100 μM) + ZnCl₂ (20 μM) | 0.02 ± 0.01 | 66 | 4 | 0.26 ± 0.04b | 866 | 5 |
| ATP (100 μM) at pH = 7.9 | 0.04 ± 0.01 | 133 | 2 | 0.18 ± 0.02b | 600 | 5 |
| ATP (100 μM) at pH = 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 ± 0.05a | 466 | 3 |
| ATP (100 μM) + high [EGTA] (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 ± 0.01a | 167 | 5 |
| ATP (100 μM) + 2APB (75 μM) | 0.02 ± 0.02 | 66 | 2 | 0.12 ± 0.04a | 400 | 3 |
| ATP (100 μM) + SKF-56-365 (50 μM) | Not tested | | | 0.25 ± 0.08b | 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.
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 P2X1, P2X2, and P2X7 using specific antibodies to those subtypes (data not shown). The P2X4 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 receptor biochemistry in cardiac tissue and myocytes (42).

An unglycosylated band was detected at ~46 kDa (the predicted molecular mass for P2X4) and a larger and broader glycosylated band at 60–65 kDa. These immunoblotting data showed that P2X4 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 P2X4 receptor channels as the major P2X receptor subtype mediating Ca2+ 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. 4D) of polarized monolayers grown on permeable supports revealed robust and apical membrane-localized expression of P2X4. 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 P2X4 receptor expression (data not shown). These data suggest that P2X4 receptors are expressed abundantly by human airway epithelial cells grown under non-polarized and polarized conditions.

The Extracellular ATP-gated P2X4 Receptor Channel Is the Major Ca2+ Entry Channel Stimulated by ATP in IB3-1 and 16HBE14o– Cells—Like other subtypes of the P2X receptor family, the P2X4 receptors are also regulated by different cations, such as H+ or Zn2+ (25). Thus, if it is true that in IB3-1 cells the prolonged Ca2+ response in Na+-free medium was due to activation of P2X4 receptors, then extracellular pH and Zn2+ should modify the ATP-induced Ca2+ signal. To test this hypothesis, we measured [Ca2+]i after changing extracellular pH or in the presence of Zn2+ 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 [Ca2+]i, only in Na+-free medium. Furthermore, in a Na+-free environment, acidic pH significantly reduced the ATP-induced peak increase in [Ca2+]i (Table II). To demonstrate directly the effect of ATP on Ca2+ influx from extracellular sources via another approach, we measured quenching of Fura-2 at 359 nm in the presence of MnCl2 (500 μM). Mn2+ is known to permeate the same entry channels as Ca2+ and quenches Fura-2 fluorescence when it enters the cells. As shown in Fig. 5A, in Na+-free medium, acidic extracellular pH (6.4) inhibited Mn2+ entry, whereas alkaline extracellular pH (7.9) potentiated markedly Mn2+ entry and quenching of the dye. To further support the involvement of P2X4 receptor channels, we tested the effect of the P2X receptor co-agonist, Zn2+, on ATP-induced Ca2+ entry mechanisms. Inclusion of ZnCl2 (20 μM) further augmented the sustained increase in [Ca2+]i induced by ATP in Na+-free medium (Fig. 5B and Table III) but had no effect in Na+-containing medium (Table III). Since our biochemical data (see above) indicated that P2X4 receptors are also present in 16HBE14o– non-CF airway epithelial cells, we tested whether increasing extracellular pH or addition of Zn2+ augmented the ATP-induced sustained Ca2+ entry in Na+-free medium in 16HBE14o– cells. As shown in Fig. 6A, ATP elicited extracellular pH-dependent quenching of Fura-2, suggesting that ATP-stimulated Ca2+ influx is facilitated by alkaline pH. In addition, similar to results obtained with IB3-1 cells, both inclusion of Zn2+ and increasing pH potentiated the effects of ATP on sustained Ca2+ signal (Fig. 6B). Taken together, these data argue for a prom-
inent role for the P2X_4 receptor as a Ca^{2+}/H_11001 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

![Graph showing pH dependence of ATP-induced Mn^{2+} entry](image)

**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+}/H_11001-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+}], in IB3-1 cells by inclusion of ZnCl_2 (compare with original trace in Fig. 2B).

![Graph showing pH dependence of ATP-induced Mn^{2+} entry](image)

**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+}], in 16HBE14o^- cells are shown in B. Effects on the sustained plateau of increased [Ca^{2+}], 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
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\(^{2+}\)/H\(^{1+}\) 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\(^{+}\)/H\(^{1+}\)-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 SOCs and TRPs could be responsible for the sustained Ca\(^{2+}\) influx induced by ATP in Na\(^{+}\)-free...
Therefore, we tested whether SOCs are present in IB3-1 cells. We treated the cells with thapsigargin (100 nM), an inhibitor of Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$ pump in the ER membrane, in the presence of extracellular Ca$_{\text{ER}}^{2+}/$H$_{\text{1001}}^{+}$. 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$_{\text{ER}}^{2+}$ plateau ($r_{\text{sustained}} = 1.58 \pm 0.29; n = 3$). In the absence of extracellular Ca$_{\text{ER}}^{2+}$, stimulation with thapsigargin resulted in a small transient increase in [Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$] due to the depletion of intracellular Ca$_{\text{ER}}^{2+}$ stores, and the re-addition of extracellular Ca$_{\text{ER}}^{2+}$ elicited a large [Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$] increase (Fig. 9). These data indicate that IB3-1 cells possess SOCs, which are activated by a decrease in [Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$].

Next, we have asked whether SOCs or store-independent TRP-like channels contribute to the sustained Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$ increase after P2Y$_1$ receptor stimulation in Na$_{\text{11001}}^{+}$-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 uM) nor SKF-56365 (50 uM) abolished the ATP-induced sustained increase in [Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$] in the absence of extracellular Na$_{\text{11001}}^{+}$ (Table III). Interestingly, the sustained Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$ 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$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$] following purinergic receptor stimulation.

**Discussion**

Stimulation of purinergic receptors exerts biological effects, which are mediated in part through elevation of intracellular Ca$_{\text{ER}}^{2+}$ 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$_S$, a specific agonist of P2Y$_1$ receptors, increased [Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$], 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$_S$ at a concentration of 100 uM may activate P2Y$_{11}$ receptors, we believe it is very unlikely that the increase in [Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$], 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$_S$ also elicited a significant increase in [Ca$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$], at a concentration of 10 uM. In other airway epithelial cell models, the presence of P2Y$_2$ has already been demonstrated (16, 58, 59). Furthermore, in *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$_{\text{ER}}^{2+}$/H$_{\text{1001}}^{+}$] in IB3-1 cells; however, both agonists do rescue impaired cell...
volume regulation in IB3-1 cells. 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 \([\text{Ca}^{2+}]_i\), an effect not observed with P2Y-specific agonists. The transient nature of the \([\text{Ca}^{2+}]_i\) signal induced by purinergic agonists accounts presumably for transient \(\text{Cl}^-\) and fluid secretion observed in different CF epithelial cell models (7, 29). Activation of P2X receptor channels under appropriate conditions would lead to \(\text{Ca}^{2+}\) influx from the extracellular space. Furthermore, this \(\text{Ca}^{2+}\) 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 P2X4.

However, our data could conceivably be explained in the following ways: 1) opening of extracellular ATP-gated P2X receptor channels; 2) activation of \(\text{Na}^+/{\text{Ca}^{2+}}\) exchanger in reverse operation mode due to \(\text{Na}^+\) removal; 3) opening of voltage-dependent \(\text{Ca}^{2+}\) channels following membrane depolarization; and 4) activation of SOCs or TRPs after depletion of intracellular \(\text{Ca}^{2+}\) stores. All lines of evidence indicate that activation of ATP-gated P2X4 receptor channels led to augmentation of \([\text{Ca}^{2+}]_i\) and the sustained \(\text{Ca}^{2+}\) plateau. First, in IB3-1 cells, BzBzATP, a P2X receptor-specific agonist, increases \([\text{Ca}^{2+}]_i\) only in \(\text{Na}^+\)-free medium. Second, the ATP-induced \(\text{Ca}^{2+}\) 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 P2Y1 receptor-specific agonist, ADP\(\beta\)S, did not cause a sustained increase in \([\text{Ca}^{2+}]_i\). Sixth, neither 2APB, an inhibitor of SOCs, nor SKF-56365, a blocker of store-operated Ca\(^{2+}\) entry, had any effect. Seventh, ATP induction of BzBzATP-dependent Ca\(^{2+}\) release was abolished in store-depleted cells, indicating 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 P2X4-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 impart 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 \(\text{Ca}^{2+}\) signal that could translate into a sustained Cl\(^-\) secretion in CF and non-CF epithelia.

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Sustained Calcium Entry through P2X Nucleotide Receptor Channels in Human Airway Epithelial Cells
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J. Biol. Chem. 2003, 278:13398-13408.
doi: 10.1074/jbc.M212277200 originally published online February 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212277200

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