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Negative Regulation of CFTR Activity by Extracellular ATP Involves P2Y2 Receptors in CFTR-expressing CHO Cells

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Abstract. Extracellular nucleotides exert autocrine/paracrine effects on ion transport by activating P2 receptors. We studied the effects of extracellular ATP and UTP on the cystic fibrosis transmembrane conductance regulator (CFTR) channel stably expressed in Chinese Hamster Ovary cells (CHO-BQ1 cells). CFTR activity was measured using the (^{125}I) iodide efflux technique and whole-cell patch-clamp recording in response to either forskolin or xanthine derivatives. Using RT-PCR and intracellular calcium concentration ($[Ca^{2+}]_i$) measurement, we showed that CHO-BQ1 cells express P2Y2 but not P2Y4 receptors. While ATP and UTP induced similar increases in $[Ca^{2+}]_i$, pre-addition by one of these two agonists desensitized the response for the other, suggesting that ATP- and UTP-induced $[Ca^{2+}]_i$ increases were mediated by a common receptor, which was identified as the P2Y2 subtype. CFTR activity was reduced by ATP and UTP but not by ADP or adenosine applications. This inhibitory effect of ATP on CFTR activity was not due to a change in cAMP level. Furthermore, CFTR activation by forskolin or IBMX failed to promote $[Ca^{2+}]_i$ increase, suggesting that CFTR activation did not generate an ATP release large enough to stimulate P2Y2 receptors. Taken together, our results show that endogenous P2Y2 receptor activation downregulates CFTR activity in a cAMP-independent manner in CHO cells.

Key words: CFTR — Intracellular Ca^{2+} — Nucleotide Receptors — Signaling pathways

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Introduction

Cystic fibrosis (CF), one of the most common lethal autosomal recessive genetic diseases, is caused by mutations in CFTR (Riordan et al., 1989), a cAMP-regulated Cl^- channel localized in the apical membrane of epithelial cells (Cheng et al., 1991). In addition to functioning as a cAMP-activated Cl^- channel, CFTR could also act as a regulator of other ion channels, such as the outwardly rectifying Cl^- channel (ORCC) (Schwiebert et al., 1995; Julien et al., 1999), the Ca^{2+}-activated Cl^- channel (CaCC) (Wei et al., 1999) or the epithelial Na^+ channel (ENaC) (Stutts et al., 1995). Several studies have suggested that a CFTR-dependent release of cellular ATP (Schwiebert et al., 1995, Urbach & Harvey, 1999) followed by the activation of P2Y nucleotide receptors could be involved in these channel regulations by CFTR. Dysfunction in CFTR activity leads to a defect in cAMP-regulated Cl^- secretion and an hyperabsorption of Na^+ through the ENaC (Kunzelmann & Mall, 2001), severely damaging the airway system and other exocrine organs such as pancreas and intestine.

One ongoing area of research suggests that among G protein-coupled receptors, P2Y nucleotide receptors are the only ones whose activation may be therapeutic in CF (Weisman et al., 1998). To date, seven mammalian P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13) have been cloned, characterized and accepted as valid members of the P2 receptor family (Ralevic & Burnstock, 1998; Communi et al., 2001; Hollopeter et al., 2001). P2Y nucleotide receptors, notably the P2Y2 subtype, are widely distributed and have been shown to be prominently localized, like CFTR, to the apical membrane of airway epithelial cells (Cressman et al., 1999, Homolya et al., 1999). In these cells, both

extracellular ATP or UTP activate the P2Y2 receptor, which is coupled to phospholipase C (PLC) via G_q protein, resulting in inositol 1, 4, 5 triphosphate (IP_3)-dependent increase in intracellular calcium concentration ($[Ca^{2+}]_i$). The P2Y2 receptor activation by ATP or UTP has been shown to stimulate Cl^- secretion through non-CFTR Ca^{2+}-activated chloride channels (CaCC) (Mason et al., 1991; Tarran et al., 2002), to inhibit Na^+ absorption mediated by ENaC (Mall et al., 2000) and to improve the mucociliary clearance (Bennett et al., 1996). These findings led to the proposal that ATP or UTP may be used as therapeutic agents for lung disease in CF epithelium in counterbalancing both the Cl^- secretion defect by activating CaCC and the hyperabsorption of Na^+ by inhibiting ENaC (Knowles et al., 1991; Mason et al., 1991; Mall et al., 2000; Kunzelmann & Mall, 2001) and thereby promoting hydration of airway secretions. Although the relationship between extracellular nucleotides and epithelial ion transport has received considerable attention, little is known about the mechanisms of regulation of epithelial ion transport by nucleotides, and direct effects of extracellular ATP on CFTR activity have been poorly studied. For example, in airway epithelial cells, extracellular ATP or UTP could stimulate CFTR-dependent anion secretion through a calcium-independent protein kinase C (PKC) pathway subsequent to the activation of the G_q-coupled P2Y2 receptor (Paradiso et al., 2001). However, in airway epithelia as in most epithelial cells the study of the effects of ATP and UTP on CFTR activity is complicated by the expression of multiple nucleotide and adenosine receptors as well as Ca^{2+}-activated Cl^- channels besides CFTR.

In this study, we have investigated further the regulatory relationship between P2Y nucleotide receptors and CFTR channels. We have sought to determine whether extracellular ATP and UTP could regulate the cAMP-evoked CFTR activity in CHO cells stably expressing human CFTR channels (Chang et al., 1998; Chappe et al., 1998). We have previously reported that CHO cells are devoid of Ca^{2+}-activated Cl^- channel activity (Chappe et al., 1998) and hence provide a *tabula rasa* for assessing the effects of nucleotides on CFTR-dependent Cl^- secretion. In addition, CHO cells do not express adenosine receptors (Iredale & Hill, 1993; Hill et al., 1997) but rather express the formerly named "P2U receptor" that is activated equally by ATP and UTP (Iredale & Hill, 1993) and is likely to correspond to the P2Y2 or P2Y4 receptor subtype. By combining the ^{125}I iodide efflux technique and the whole-cell variant of the patch-clamp technique together with RT-PCR, molecular cloning and intracellular Ca^{2+} monitoring, we show that CHO cells express functional P2Y2 but not P2Y4 receptors and that stimulation of this particular receptor subtype by ATP or UTP decreases cAMP-dependent CFTR activation. To our knowledge, this is the first report of repression of CFTR activity by P2Y receptors.

Materials and Methods

Solutions and Chemicals

Fura2-AM was purchased from Molecular Probes (Netherlands). Adenosine-5′-triphosphate (ATP), uridine-5′-triphosphate (UTP), KN-62 (1-[N,O-bis(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine), IBMX (1-methyl-3-isobutyl xanthine), DPMX (7-methyl-1, 3-dipropyl xanthine) and other chemicals were purchased from Sigma (SL. Louis, MI). Forskolin was dissolved in ethanol, Fura2-AM, IBMX and DPMX were dissolved in dimethyl sulfoxide (DMSO). The final concentration of ethanol or DMSO in the experiments was less than 0.1% and was found to have no significant effect on iodide efflux. Culture medium, serum, penicillin and streptomycin were purchased from Bio Media (Boussens, France).

Cell Culture

Cells were grown in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°C. CHO cells, stably transfected with pNUT vector containing wild-type CFTR (CHO-BQ1), were provided by J. R. Riordan and X. B. Chang, Scottsdale, AZ, USA (Chang et al., 1998). Cells were maintained in αMEM supplemented with 7.5% fetal calf serum, 2 mM glutamine, penicillin (50 IU/ml), streptomycin (50 μg/ml) and methotrexate (100 μM).

RT-PCR and Molecular Cloning

Total RNA was extracted from CHO-BQ1 cells using Tri Reagent (Euromedex, France) according to the manufacturer's instructions. The RNA was then quantified by spectrophotometry, aliquoted in sterile water and stored at −80°C. First-strand cDNA synthesis was performed with 4 μg of total RNA using the SuperScript preamplification system (Life Technologies, France). To rule out any genomic DNA contamination, total RNA was incubated with RQ1 DNase-RNase free (Promega, France), and experiments were also performed without reverse transcriptase (RT) to control for the cDNA origin of the amplified fragment. To amplify the P2Y2 receptor cDNA, a set of degenerate primers (P2Y2 sense primer, 5′-AG(G,C)-ATC-CTC-TTC-CTC-A(A,C)C-TGC-ATC-AG-3′ and the P2Y2 antisense primer, 5′- GGG-TCA-AG(A,G)-CAA-CTG-TT(A,G)-GC(A,G)-CTG-GC-3′) were designed from highly conserved regions between human (GenBank™ accession number Gi:4505558), mouse (GenBank™ accession number Gi:6679194) and rat (GenBank™ accession number Gi:1336124) P2Y2 genes (Lustig et al., 1993; Parr et al., 1994; Communi et al., 1995; Rice, Burton & Fiedeldey, 1995; Chen et al., 1996; Seye et al., 1997), and synthesized (Eurogentec, Belgium). Similarly, a set of specific primers (P2Y4 sense primer, 5′-TGG-CAT-TGT-CAG-ACA-CCT-TGT-ATG-TG-3′ and the P2Y4 antisense primer, 5′-AAG-CAG-ACA-GCA-AAG-ACA-GTC-AGC-AC-3′) were designed for the amplification of the P2Y4 purinergic receptor cDNA, based on sequence homology between human (GenBank™ accession number Gi:4505560) and rat (GenBank™ accession number Gi:13928943) P2Y4 receptors (Communi et al., 1995; Webb et al., 1998).

After reverse transcription, 2 μl of the first-strand synthesis product were directly amplified by PCR in a 50 μl reaction, using the P2Y2 degenerate primers (500 pM each) or the P2Y4 specific

primers (500 pM each) with 2.5 U of the Taq DNA polymerase (Promega, France) in 1.5 mM $MgCl_2$. The PCR amplification (Robocycler Stratagene, Europe) conditions were: 94°C for 5 min, 1 cycle; 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 40 cycles; 72°C for 4 min, 1 cycle. PCR products were analyzed using 1.5% agarose gel electrophoresis and ethidium bromide coloration, and extracted from gel using Millipore ultrafree-MC centrifugal filter tube. The PCR amplification product obtained with the P2Y2 degenerate primers was cloned using the pGEM-T Easy Vector Systems (Promega, France), the presence of inserts was confirmed by restriction analysis and DNA inserts were sequenced (Eurogentec, Belgium). As a positive control, the same sets of primers were used to amplify rat and hamster genomic DNA, and PCR products were cloned and sequenced. As a negative control, PCR was carried out as before, except that water was added instead of DNA template.

Sequence Analysis

DNA and amino acid sequences obtained were identified, analyzed and compared with sequences compiled in different banks using the computer programs BLAST (Altschul et al., 1990), FASTA (Pearson & Lipman, 1988) and ClustalW (version 1.8) on the infobiogen site (http://www.infobiogen.fr).

Measurement of Intracellular Free Calcium

Cells were cultured four days on glass coverslips. Confluent cell monolayers were loaded with 2.5 μM fura-2/AM for 1 h at 37°C in serum-free DMEM/F12 medium, then washed twice at room temperature with modified Earle's salt solution (B medium) containing (in mM): 137 NaCl, 5.36 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 10 HEPES-NaOH, pH 7.4. Cells were then placed into the open-topped circular microperfusion chamber (200 μl bath volume) and mounted on an OLYMPUS IX50 fluorescent inverted microscope, equipped with a 20× lens. For each experiment a new glass coverslip was used. Cells were superfused with test solutions 1 ml/min until no further change in Ca^{2+} signal was detected. Each test solution was freshly prepared and applied in the syringe linked to the chamber with a manifold. The light from a xenon lamp (USHIO) was filtered through alternating 340- and 380-nm interference filters under computer control (TILLvisION system, Germany). The emission fluorescence produced after fura-2 excitation, was passed through a 400-nm dichroic mirror, filtered at 510 nm. Cells were sequentially illuminated (50 ms) at 340 and 380nm and the fluorescence emission (510nm) was monitored via a 12 bit intensified CCD video camera (IMAGO, T.I.L.L. Photonics, Germany) coupled to the microscope. Standard curves used to calculate calcium levels were obtained using a range of EGTA-buffered calcium solutions of the fura-2 free acid. $[Ca^{2+}]_i$ was calculated using the formula proposed by Grynkiewicz (Grynkiewicz, Poemie & Tsien, 1985): $[Ca^{2+}]_i = K'(R-R_{min}/R_{max}-R)$, where K′ is the product of the dissociation constant of Ca^{2+} binding with fura-2, R is the experimental ratio of F_{340} and F_{380} from which the background fluorescence has been subtracted, and R_{max} and R_{min} are the values of R under saturating (5 μM ionomycin) and Ca^{2+}-free conditions (4 mM EGTA combined with 5 μM ionomycin), respectively. Thus, $[Ca^{2+}]_i$ of individual cells was calculated every 5 seconds and values were plotted versus time. For each experiment (except as otherwise indicated) fifty cells were arbitrarily selected in the field of view and $[Ca^{2+}]_i$ was averaged.

Agonists' concentration-response curves were fitted using the Hill equation $Y = y_{min} + (y_{max}-y_{min}) / ((1 + 10^{logEC50\text{-}X}\cdot n_H))$ and the half maximal effect (EC_{50}) values were calculated using GraphPad Prism v3.0 (GraphPad Software), where Y is the response, y starts at the bottom (y_{min}) and goes to the top (y_{max}), X is the logarithm of concentration and n_H is the Hill coefficient.

Measurement of Cellular cAMP

CHO cells grown for four days in 12-well plates were washed twice with 2 ml of B medium, then 0.5 ml of this buffer containing the drug to be tested were added to each well. After a 5 min incubation period at 37°C, the reaction was stopped by addition of 55 μl of 11 N perchloric acid. Cells were frozen for later cAMP determination using RIANEN kit (New England Nuclear). cAMP levels were normalized to total protein and expressed as pmoles/mg of protein accumulated in 5 min ± SD.

Determination of Iodide Efflux

Cells were cultured for four days in 24-well culture plates. Before experiments, the culture medium was removed and cells were washed twice with 500 μl of B medium (*see* above for composition). Cells were then loaded in B medium containing 1 μM KI and 0.5 μCi of ^{125}INa/ml for 30 min. After loading, cells were washed four times with 500 μl of iodide-free B medium. 500 μl of B medium were then added and removed sequentially every 30 s for 2.5 min. Agonists or antagonists to be tested were added at time zero of the efflux. At the end of the efflux, intracellular ions were extracted by the addition of 1 ml trichloroacetic acid (7.5%) to the cell layer. All samples were counted using a γ counter (Kontron). Precipitated proteins were solubilized in 0.1 N NaOH and quantified using Coomassie protein Assay Reagent (Pierce, USA).

Data Analysis

Tracer contained in the cell layer at the onset of the efflux was calculated as the sum of samples and extract count. Efflux curves were constructed by plotting the percent of cell layer content ($I\%$) remaining in the cell layer versus time. Rate constants (k, min^{-1}) from unstimulated or stimulated efflux were determined by fitting efflux curves to mono-exponential function $I\% = 100\ \exp(-kt)$ using linear regression of the neperian logarithm of the data. k is used in Results to calculate iodide accumulated in the medium versus time. We hypothesized that, in the presence of a stimulator, the efflux was the sum of two iodide effluxes occurring in parallel: a basal efflux and a stimulated efflux characterized by the rate constants k_b and k_s, respectively. The net total efflux was therefore described by $I_t\% = 100\ \exp(-k_t t)$ where k_t is the sum of k_b and k_s rate constants. Finally, k_s calculated as $k_t - k_b$ was used to establish concentration-response relationships for agonists and antagonists. Data are expressed as means ± SD, and t test was used to determine significance. Agonists' and antagonists' concentration-response curves were fitted using the hyperbolic equation $Y = y_{max}\cdot X/(EC_{50} + X)$, where Y is the response, y starts at the bottom and goes to the top (y_{max}), X is the concentration and the half maximal effect (EC_{50}) values were calculated using GraphPad Prism v3.0 (GraphPad Software), and the Hill equation described above, respectively.

Whole-cell Patch-clamp Recording

CHO-BQ1 cells were plated on 35-mm Petri dishes and cultured at 37°C in 5% CO_2 for four days before use. Currents were recorded using the patch-ruptured whole-cell variant of the patch-clamp technique. The external solution consisted of (mM): 120 NaCl, 23 $NaHCO_3$, 3 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, 11 glucose (bubbled with a 95% O_2-5% CO_2 mixture, pH 7.4). The intracellular solution was (mM) KCl, 130; $MgCl_2$, 1; MgATP, 4; Na_3GTP, 0.2; 11 EGTA; 0.5 $CaCl_2$; HEPES, 10 mM (adjusted to pH 7.3 with KOH, 295 mOsmol/L). When filled with this internal solution, pipette resistance was 5 MΩ. Currents were measured with an Axopatch 200B amplifier and filtered at 2 kHz. Cell membrane capacitance and series resistance

compensations were applied (75–85%). The voltage-current relationships were determined using slow voltage ramps or step voltage protocols. Experiments were performed at room temperature and drugs were applied by using a gravity-fed perfusion system at 5 $ml \cdot min^{-1}$. Data were expressed as the mean ± SE of the mean and Student's *t*-test was used to determine the statistical significance and differences were considered significant if $P < 0.05$.

Results

Detection of P2Y2 but not P2Y4 Transcripts in CHO-BQ1 Cells

Pharmacological experiments have previously shown that "P2U receptors" constitutively expressed in CHO-K1 cells are equally activated by ATP and UTP (Iredale & Hill, 1993). Although this pharmacology would be consistent with a contribution by P2Y2 and/or P2Y4 receptors (Ralevic & Burnstock, 1998), the receptor subtypes involved in these responses have not been identified. We therefore used RT-PCR and subsequent molecular sequencing of the RT-PCR products to test for the expression of P2Y2 and P2Y4 transcripts in CHO-BQ1 cells.

As no hamster P2Y receptor sequences have been cloned, we used degenerate primers designed to recognize highly conserved regions in the second intracellular loop and the third extracellular loop of P2Y2 receptors from human, mouse and rat (Lustig et al., 1993; Parr et al., 1994; Communi et al., 1995; Rice et al., 1995; Chen et al., 1996; Seye et al., 1997). The PCR product obtained from CHO-BQ1 cell cDNA (a single band of 550 bp) (Fig. 1*A*, *B*) was subcloned and fifteen clones were sequenced. A unique cDNA sequence was obtained, which shared 91% and 89% homology with mouse and rat P2Y2 receptor cDNA, respectively and 98% and 96% identity with mouse and rat deduced amino-acid sequence, respectively (*data not shown*) (GenBank™ accession number AF314034).

No RT-PCR product was obtained from CHO-BQ1 cells using specific P2Y4 primers (*see Methods*), whereas a single band of 543 bp was amplified with the same set of P2Y4 primers from genomic DNA as positive control (Fig. 1*C*). The PCR amplification product was sequenced (GenBank™ accession number AF453823) and showed 92% homology with both mouse and rat P2Y4 receptor genes (*data not shown*) (Webb et al., 1998; Suarez-Huerta et al., 2001). These results indicate that CHO-BQ1 cells expressed P2Y2 but not P2Y4 receptor mRNAs.

Effect of ATP and UTP on Intracellular Calcium Concentration ($[Ca^{2+}]_i$)

To assess the functional expression of P2Y2 receptors in CHO-BQ1 cells we used fura-2 fluorescence calcium imaging. Ratiometric fluorescence (340/380) was used to calculate $[Ca^{2+}]_i$ according to Grynkiewicz et al. (1985). For each experiment, fifty cells were individually analyzed and $[Ca^{2+}]_i$ averaged and plotted versus time as described in Methods.

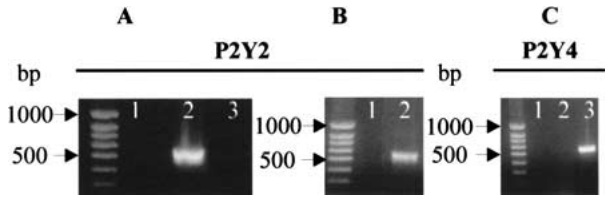

Fig. 1. Detection of P2Y2 but not P2Y4 mRNAs in CHO-BQ1 cells by RT-PCR experiments. The extraction of total RNA and the amplification by RT-PCR were performed as described in Methods. Amplification with P2Y2 degenerate primers resulted in a single band of a 550-bp product (*A*, lane *2*). No amplification product was detected with P2Y4 specific primers (*C*, lane *1*). As positive control, PCRs were performed using P2Y2 and P2Y4 sets of primers from hamster genomic DNA. A single band for P2Y2 (*B*, lane *2*) and P2Y4 (*C*, lane *3*) of 550- and 543 bp, respectively, was obtained and sequenced. Negative controls were carried out for RT (*A*, lane *1* and *C*, lane *1*) and PCR (*B*, lane *1* and *C*, lane *2*) experiments.

Concentration-Effect Relationship for ATP and UTP on $[Ca^{2+}]_i$

For each experiment, the resting $[Ca^{2+}]_i$ in unstimulated cells was determined for 3 min and averaged. Figure 2 shows the effect of increasing concentrations of ATP and UTP on $[Ca^{2+}]_i$. ATP at 1 μM exhibited a sharp and rapid Ca^{2+} response (Fig. 2*A*) only in ~30 % of the cells in the field. Moreover, at this concentration the cells were responsive in an asynchronous manner (*data not shown*). Synchronism of the Ca^{2+} response was obtained for concentrations of ATP above 3 μM (*data not shown*). ATP at 10 μM induced synchronous and maximal Ca^{2+} responses (Fig. 2*B*) in most of the cells. The same typical responses were observed for UTP (*data not shown*). The change in Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) in response to nucleotide application (*see* Methods) was calculated by subtracting the resting $[Ca^{2+}]_i$ from maximal $[Ca^{2+}]_i$. The amplitude of the Ca^{2+} response to both ATP and UTP (Fig. 2*C*) was found to be concentration-dependent with half-maximal effective concentration (EC_{50}) values of 2.1 ± 1.1 μM and 1.9 ± 1.4 μM, respectively.

Role of Extracellular Ca^{2+} in the ATP- and UTP-induced Changes in $[Ca^{2+}]_i$

ATP (10 μM) caused a transient rise in $[Ca^{2+}]_i$, with a mean $\Delta[Ca^{2+}]_i$ of 78.6 ± 16.5 nM ($n = 8$), that displayed a biphasic time course with a fast transient peak followed by a plateau (Fig. 3*A*). Basal level of $[Ca^{2+}]_i$ recovered within 6 ± 2 min ($n = 8$). In the absence of extracellular Ca^{2+}, the plateau phase, but

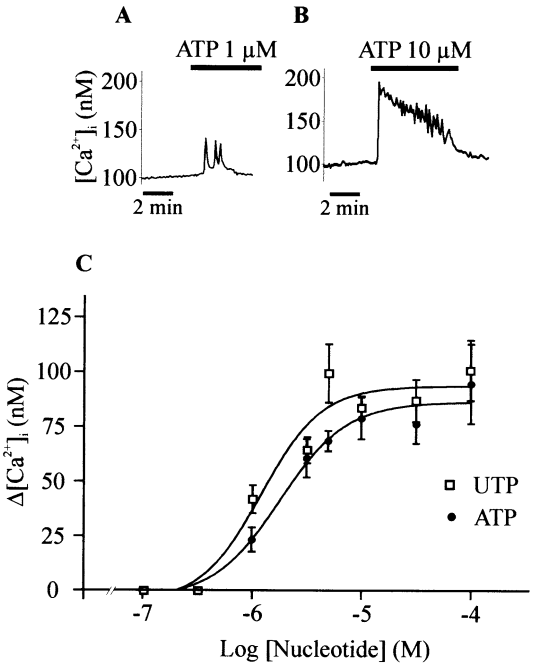

Fig. 2. Concentration-effect relationship for ATP- and UTP-stimulated Ca^{2+} responses. (*A*) and (*B*) represent typical single-cell Ca^{2+} responses induced either by 1 μM (*A*) or 10 μM (*B*) ATP. The bar above each trace indicates the time of drug application. (*C*) Concentration-response relationship for ATP and UTP. Curves were fitted using the Hill equation (*see* Methods for details). Data are means ± SEM, for *n* independent experiments (*n* = 3–8).

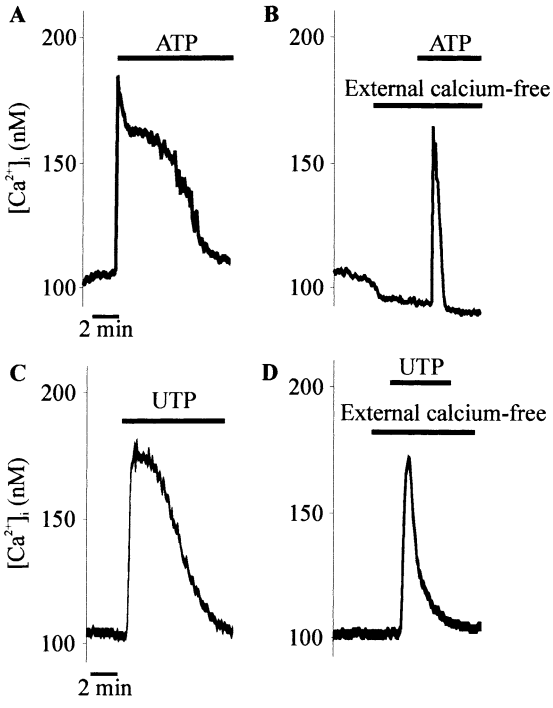

Fig. 3. Role of extracellular Ca^{2+} in ATP- and UTP-induced Ca^{2+} responses. (*A*) and (*C*) show the Ca^{2+} responses for ATP and UTP (10 μM each) in the presence of extracellular Ca^{2+}. (*B*) and (*D*) show the effects of the removal of extracellular Ca^{2+} on the time course of the Ca^{2+} signal induced by both ATP and UTP. Traces shown are representative of *n* independent experiments (*n* = 3–8).

not the fast peak of the response obtained with 10 μM ATP, was abolished (*n* = 5) (Fig. 3*B*), indicating that the first rise in $[Ca^{2+}]_i$ was due to Ca^{2+} release from intracellular stores and the subsequent rise due to Ca^{2+} influx across the plasma membrane.

Figures 3*C, D* and Fig. 4*A* show the effect of UTP on $[Ca^{2+}]_i$. Addition of UTP (10 μM) produced a Ca^{2+} response with a $\Delta[Ca^{2+}]_i$ of 83.0 ± 8.9 nM (*n* = 4) and basal level was recovered after 7 ± 2 min. As observed for ATP, the plateau phase in response to UTP (10 μM) was absent when the extracellular Ca^{2+} was removed.

Evidence that ATP and UTP Act at the Same Receptor

By contrast to ATP and UTP, addition of UDP (10 μM) failed to promote a rise in $[Ca^{2+}]_i$ (Fig. 4*A*) indicating that UTP-induced Ca^{2+} response is not due to the UTP hydrolysis product UDP, known to activate the G_q-protein coupled P2Y6 receptor (Communi, Parmentier & Boeynaems, 1996).

In order to determine whether ATP and UTP act at the same receptor, we have investigated cross-desensitization between these two agonists. Cells were first stimulated with a given agonist; then, when no further change in Ca^{2+} signal was detected, cells were stimulated with a second agonist in the continued presence of the first one. As shown in Fig. 4*A*, UTP pretreatment abolished the ATP-induced Ca^{2+} response and pre-addition of ATP markedly decreased the Ca^{2+} response to UTP, indicating a cross-desensitization in the effects of ATP and UTP. Taken together, these results suggest that CHO-BQ1 cells expressed a common receptor for ATP and UTP that has the pharmacological features of the P2Y2 subtype.

To test for an involvement of the endogenous P2X7 receptor (Michel et al., 1998), we used the P2X7 inhibitor, KN-62 (50 nM) (Chessel, Michel & Humphrey, 1998; Baraldi et al., 2000) on the ATP-induced Ca^{2+} response. No significant modification of the amplitude ($\Delta[Ca^{2+}]_i$ of 65.6 ± 14.0 nM; *n* = 4; *t*-test: $P > 0.05$) and the duration (6 ± 2 min) of the ATP response was observed after treatment of the cells with this inhibitor (Fig. 4*B*).

In addition, pertussis toxin (PTX) pretreatment (100 ng/ml) for 24 h, a compound that prevents $G_{i/o}$ protein activation of effectors, did not change either basal $[Ca^{2+}]_i$ or the number of responsive cells, and had no significant effects (*t*-test: $P > 0.05$) on both ATP (10 μM; *n* = 3)- and UTP (10 μM; *n* = 3)-induced Ca^{2+} responses (Fig. 4*C*), suggesting that

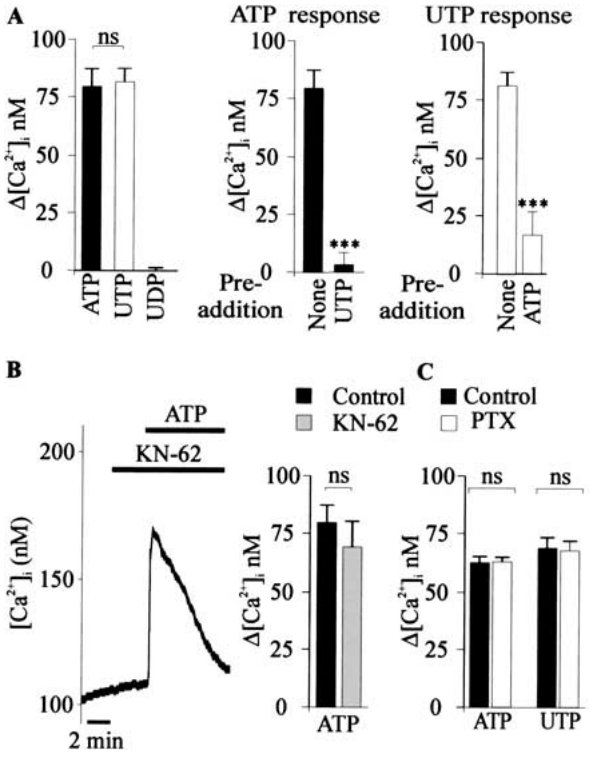

Fig. 4. ATP and UTP act at the same receptor. (*A*) Cross-desensitization between ATP- and UTP-stimulated Ca^{2+} responses. Ca^{2+} responses induced by ATP, UTP or UDP (10 μM each) were measured following no pre-addition (*None*) or pre-addition of 10 μM of the nucleotides as indicated (n = 3–8). (*B*) Effect of KN-62 (50 nM) treatment on ATP (10 μM)-induced Ca^{2+} response. The trace and values are representative of n independent experiments (n = 4). (*C*) Effect of PTX treatment (100 ng/ml, for 24 hr) on ATP- (10 μM; n = 3) and UTP- (10 μM; n = 3) induced Ca^{2+} responses. The values are means ± SEM (***, $P < 0.001$; *ns*, not significant, $P > 0.05$; *t*-test).

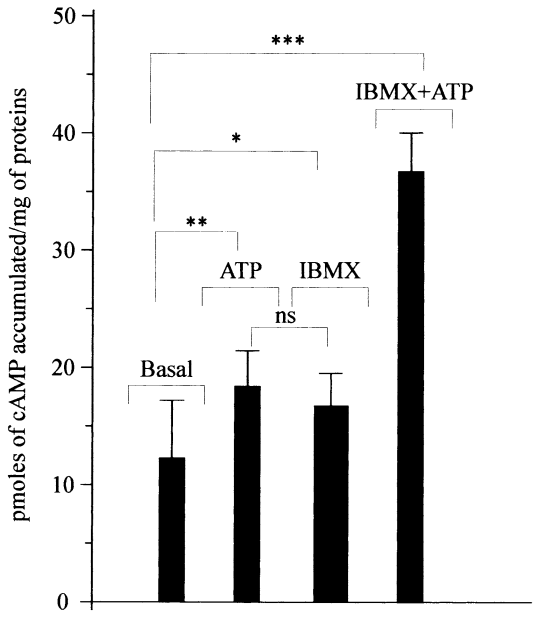

Fig. 5. Effect of P2Y2 receptor-mediated regulation on cellular cAMP content. The cAMP level was determined as described in Methods. cAMP content was measured in resting cells (Basal; n = 17) or in the presence of ATP (100 μM, n = 8), IBMX (50 μM, n = 8) or with the simultaneous addition of ATP (100 μM) and IBMX (50 μM) (n = 4). Data are expressed as pmoles of cAMP per mg of proteins accumulated in 5 min and represent means ± SD for n experiments (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; *ns*, not significant; *t*-test).

P2Y2 receptors couple to G_q-type G proteins in CHO-BQ1 cells. Furthermore, no effect of PTX was also observed on Ca^{2+} responses for ATP concentrations from 2 to 100 μM (*data not shown*).

These observations, together with molecular sequencing, suggest that the P2Y2 receptor is functionally expressed in CHO-BQ1 cells and accounts for ATP- and UTP-induced Ca^{2+} responses.

ATP Induces cAMP Accumulation in CHO-BQ1 Cells

It has been reported that P2Y receptor activation can regulate intracellular cAMP level (Ralevic & Burnstock, 1998; Zambon et al., 2001), and thereby could modulate CFTR activity. We therefore measured the cellular cAMP content in response to P2Y2 receptor stimulation. Basal level of cAMP (12.25 ± 4.5 pmol/mg of proteins; n = 17) was slightly, but significantly, increased by 5 min application of either 100 μM ATP (18.4 ± 3.0 pmol/mg; n = 8) or 50 μM IBMX (an inhibitor of phosphodiesterase) (16.7 ± 2.8 pmol/mg; n = 8) (Fig. 5). Combined application of ATP (100 μM) with IBMX (50 μM) for 5 min raised cAMP level up to 36.8 ± 3.1 pmol/mg (n = 4), 19% of the activity induced by 1 μM forskolin (FSK; 193.7 ± 30 pmol/mg; n = 4).

Extracellular ATP Inhibits FSK-induced CFTR Activation

We have previously used the ^{125}I iodide efflux technique together with electrophysiological methods to characterize the specific activation of CFTR chloride channels by cAMP agonists and xanthine derivatives in CHO-BQ1 cells (Chappe et al., 1998). We have established that forskolin stimulated ^{125}I efflux in CFTR-expressing CHO cells but not in parental (CHO-K1) and mock-transfected (CHO-KNUT) cells. Forskolin-, IBMX- and DPMX-stimulated ^{125}I effluxes were significantly inhibited by glibenclamide (100 μM) and were insensitive to DIDS (100 μM), indicating that CFTR was the only Cl^- channel activated by these compounds in CFTR-expressing CHO cells. Furthermore, no Ca^{2+}-activated ^{125}I iodide efflux has been detected in these cells (Chappe et al., 1998). A subsequent paper validated the iodide efflux technique as a suitable method to monitor CFTR activity in these cells (Becq et al., 1999).

In the present experiments, we used ^{125}I efflux rates (k, min^{-1} together with the patch-clamp technique to quantify CFTR activation. The constants k_b, k_s and k_t refer to the basal, stimulated and total efflux rates (min^{-1}), respectively, as described in Methods. Figure 6*A* shows the 1 μM forskolin-stimulated efflux (k_t = 0.81 ± 0.07 min^{-1}; n = 12) and the unstimulated basal efflux (k_b = 0.18 ± 0.01 min^{-1}; n = 12). In the presence of ATP (100 μM) the 1 μM forskolin-stimulated efflux was reduced to kt = 0.47 ± 0.1 min^{-1} (n = 12) while the basal efflux was unchanged (k_b = 0.19 ± 0.01 min^{-1}; n = 12). The curves are expressed as percent of the initial cellular iodide accumulated in the medium versus time. Figure 6*B* represents the effect of increasing concentrations of FSK on the stimulated efflux rate. Maximal efflux rate was obtained at 3 μM FSK (k_t = 1.26 ± 0.07 min^{-1}; n = 9). No detectable stimulated efflux was observed for 0.1 μM FSK and half-maximal stimulation required 0.6 ± 0.16 μM of FSK (k_t for 1 μM FSK = 0.81 ± 0.06 min^{-1}, n = 18). Addition of ATP (100 μM) in the presence of increasing concentrations of FSK significantly reduced the amplitude of the FSK-induced ^{125}I efflux. In the presence of ATP, k_t obtained with 10 μM FSK was reduced to 0.8 ± 0.18 min^{-1} (n = 9) (~60% of the initial value) while the concentration of FSK needed to obtain half-maximal stimulation (EC_{50}) shifted from 0.6 ± 0.16 μM in the absence of ATP to 0.8 ± 0.4 μM in the presence of ATP.

We further tested the effects of ATP on CFTR activity using whole-cell recording of CHO-BQ1 cells (Fig. 6*C–E*). The whole-cell current-voltage (*I-V*) relationships were studied in intracellular and extracellular solutions containing equal concentrations of Cl^-. ATP, applied at 100 μM for 60 s, had no significant effect on the whole-cell membrane conductance of CHO-BQ1 cells (3.3 ± 0.2 and 3.5 ± 0.3 nS in the absence or presence of ATP, respectively) (Fig. 6*C, E*). Bath application of forskolin produced a 10-fold increase in membrane conductance (30.2 ± 3 nS, n = 5) (Fig. 6*D, E*). The reversal potential of the FSK-induced current was 1 ± 0.6 mV, showing that Cl^- was the major ion contributing to this current and indicating that CFTR is functionally expressed in CHO-BQ1 cells, as demonstrated previously (Jia et al., 1997). Applying ATP (100 μM) decreased the FSK-induced CFTR current density by 49%, reducing the whole-cell membrane conductance from 30.2 ± 3 nS to 19 ± 2 nS (n = 5) (Fig. 6*D, E*).

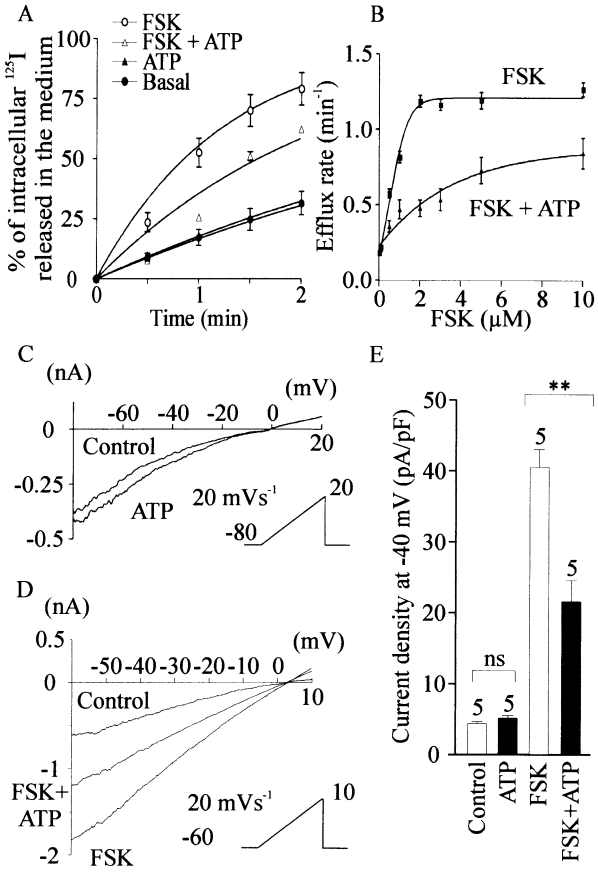

Fig. 6. Effect of extracellular ATP on CFTR activity. (*A*) Effect of addition of FSK on stimulated ^{125}I efflux. ^{125}I efflux kinetics were measured in the absence (*filled symbols*) or presence (*empty symbols*) of FSK (1 μM) with or without ATP (100 μM). Results are expressed as percent of intracellular ^{125}I released in the medium. Data are means ± SD for n = 12. Solid curves are exponential efflux rates for both basal and 1 μM FSK-stimulated efflux. (*B*) Concentration-dependent activation of ^{125}I efflux for FSK alone (*control*) or in the presence of ATP (100 μM). The rate constant (k_t) from ^{125}I efflux was measured in the presence of increasing concentrations of FSK alone (*control*) or in the presence of ATP (100 μM). Curves were fitted using the hyperbolic equation described in Methods. Data represent means ± SD for n = 3–9. (*C–E*) Inhibition of CFTR currents by ATP in CHO-BQ1 cells. (*C*) representative steady-state *I-V* relationships in a CHO-BQ1 cell in the presence or absence of ATP (100 μM). Whole-cell currents were evoked by slow voltage ramps as indicated in inset. (*D*) Representative *I-V* relationships obtained in a CHO-BQ1 cell in control conditions and upon bath application of FSK (1 μM) or FSK (1 μM) plus ATP (100 μM). (*E*) summary of current densities (pA/pF) recorded at −40 mV in unstimulated CHO cells (*control*) and in cells exposed to FSK (1 μM) with and without ATP (100 μM). Bars represent means ± SEM for the number of cells indicated (**, $P < 0.01$; paired t-test).

Extracellular ATP Inhibits Xanthine Derivative-induced CFTR Activation

We tested the effect of ATP on CFTR activation induced by the xanthine derivatives IBMX and DPMX. IBMX and DPMX were was found to be less efficient than forskolin in promoting maximal iodide efflux (Chappe et al., 1998). Figure 7*A* shows that the effluxes induced either by IBMX (n = 8) or DPMX (n = 4) were lowered by ~40% of initial values in the presence of ATP (100 μM).

ATP and UTP Cause Similar Inhibition of CFTR Activity: Concentration-Effect Relationship

Figure 7*B* represents the concentration-effect relationship for ATP and UTP on FSK (1 μM)-stimulated

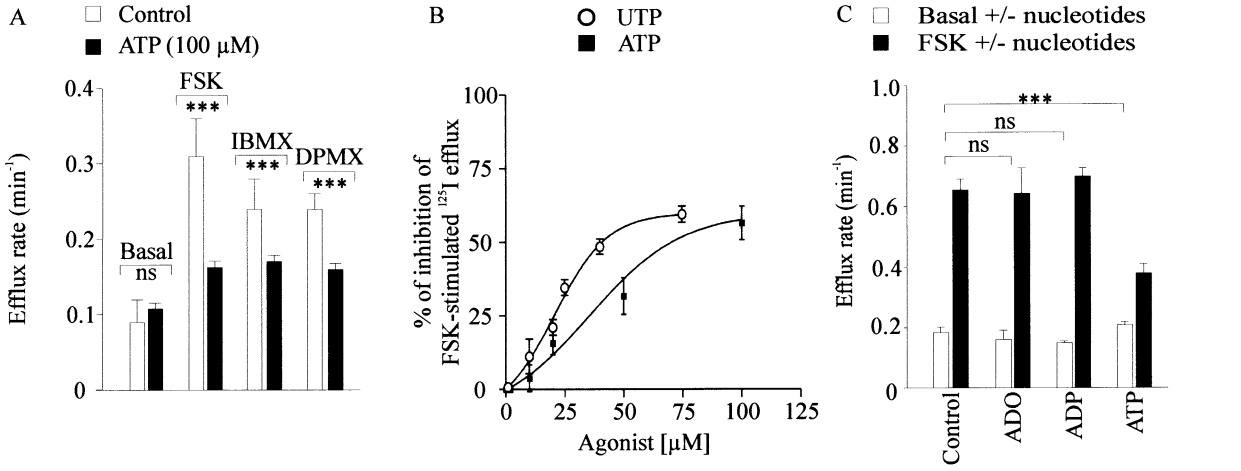

Fig. 7. (*A*) Inhibition of FSK- and xanthine-activated CFTR by external ATP (100 μM). Iodide efflux was measured in the absence (Basal, $n = 6$) or in presence of FSK (1 μM, $n = 8$), IBMX (100 μM, $n = 8$) or DPMX (100 μM, $n = 4$) and with (*black bars*) or without (*white bars*) ATP (100 μM). (*B*) Concentration-dependent inhibition of FSK-stimulated ^{125}I efflux by external ATP and UTP. The basal efflux rate (k_b) was subtracted from k_t to obtain the stimulated efflux rate $k_s = k_t - k_b$. The rate constant from basal (k_b) and FSK (1 μM) (k_s) stimulated ^{125}I efflux was measured in the presence of increasing concentrations of ATP and UTP. Data represent means ± SD for $n = 3$, and were fitted using the Hill equation. (*C*) Effect of adenosine (ADO) and ADP on FSK-activated CFTR. Iodide efflux was measured in the absence (Basal, $n = 6$) (*white bars*) or in the presence of forskolin (1 μM, $n = 8$) (*black bars*) and with or without adenosine, ADP, ATP or UTP (100 μM) as indicated below the bars on the figure. Data represent means ± SD for $n = 3$–9 (***, $P < 0.001$; *ns*, not significant; *t*-test).

^{125}I efflux. The FSK (1 μM)-stimulated ^{125}I efflux ($k_s = 0.5 \pm 0.05$ min^{-1}) was reduced by 56.5% ± 9.9 and 58.8% ± 4.8 in the presence of ATP (100 μM) and UTP (75 μM) respectively. Half-maximal inhibitory effect was reached with approximately 35 μM ATP and 25 μM UTP. No significant modification was seen on basal efflux in the presence of increasing concentrations of ATP or UTP.

Effect of ATP Metabolites as Putative Purinergic Agonists on CFTR Activity

We further examined whether the ATP-induced CFTR inhibition was due to the direct action of ATP or to one of its metabolites, ADP or adenosine. As shown in Fig. 7*C*, neither adenosine nor ADP had significant effects either on CFTR activity or on basal ^{125}I efflux, suggesting that ATP-induced CFTR inhibition was not due to an indirect effect of ADP or adenosine.

Effect of Ca^{2+} Ionophore A23187 on FSK-induced CFTR Activation

We examined whether a transient increase in $[Ca^{2+}]_i$ could account for the ATP-induced CFTR inhibition. Figure 8*A* shows that the application of the Ca^{2+} ionophore A23187 (2 μM) produced a transient (~2 min) and monophasic increase in $[Ca^{2+}]_i$. A23187 (2 μM), which had a small effect (*t*-test: $P < 0.05$) on basal efflux rate, failed to inhibit the FSK (1 μM)-induced CFTR activation (Fig. 8*B*).

CFTR Stimulation Fails to Induce $[Ca^{2+}]_i$ Increase and to Modify ATP-induced Ca^{2+} Response

It has been suggested that in CHO cells CFTR activation is able to modulate $[Ca^{2+}]_i$ by promoting ATP release (Urbach & Harvey, 1999; *see*, however, Grygorczyk, Tabcharani & Hanrahan, 1996). To test whether activation of CFTR may promote ATP release and thereby activates P2Y2 receptors, we monitored any changes in $[Ca^{2+}]_i$ that could occur during IBMX and FSK stimulation. Figure 9 shows that neither IBMX (100–250 μM; $n = 4$) nor FSK (5–20 μM; $n = 5$) changed basal $[Ca^{2+}]_i$. Moreover, when cells were treated with IBMX (250 μM) or FSK (10 μM), the ATP-induced Ca^{2+} response was not significantly changed (*t*-test: $P > 0.05$) in comparison with the Ca^{2+} response induced by ATP alone ($\Delta[Ca^{2+}]_i$ of 72.7 ± 24.2 nM, $n = 3$; 57.1 ± 13.4 nM, $n = 3$; 78.6 ± 16.5 nM, $n = 8$, respectively). In addition, the basal level was recovered in each condition within 6 ± 2 min (*data not shown*).

These data suggest that in these cells, CFTR activation did not promote an ATP release sufficient to increase $[Ca^{2+}]_i$ and did not change the P2Y2-induced Ca^{2+} response.

Discussion

The present results demonstrate for the first time that, in a heterologous model, CFTR activity is inhibited

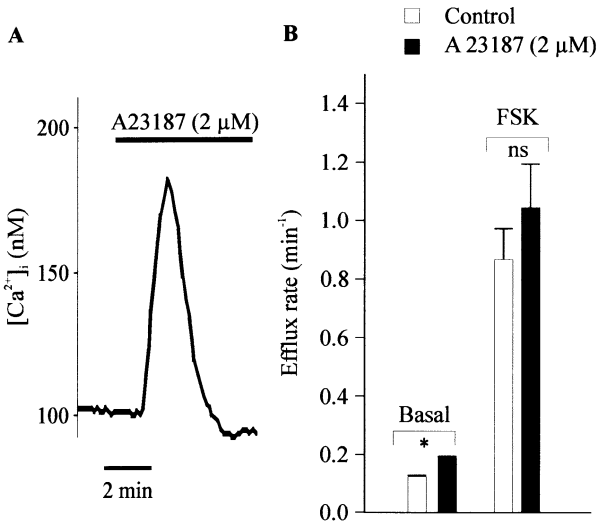

Fig. 8. Role of Ca^{2+} in CFTR activity. (*A*) Effect of A23187 (2 μM) on $[Ca^{2+}]_i$. This trace is representative of three independent experiments. (*B*) Effect of A23187 (2 μM) on forskolin-activated CFTR. Iodide efflux was measured in the absence (Basal, $n = 3$) or in presence of FSK (1 μM, $n = 3$) and with (*black bars*) or without (*white bars*) A23187 (2 μM, $n = 3$) (*, $P < 0.05$; *ns*, not significant; *t*-test).

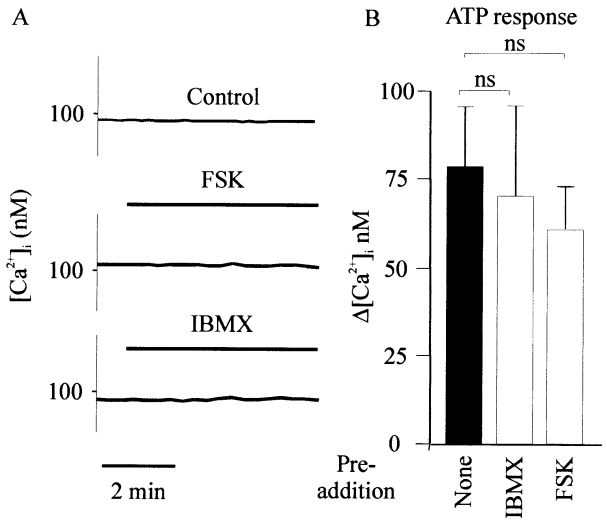

Fig. 9. (*A*) Effects of FSK (10 μM) and IBMX (250 μM) on basal $[Ca^{2+}]_i$. Traces are representative of five (IBMX) and four (FSK) independent experiments. (*B*) Effects of FSK and IBMX treatment on ATP-induced $[Ca^{2+}]_i$ increase. Ca^{2+} responses induced by 10 μM ATP were measured following no preaddition *(None)* or pre-addition of FSK (10 μM) or IBMX (250 μM) as indicated. The values are means ± SEM ($n = 3$–8) (*ns*, not significant; *t*-test).

by P2Y2 receptors in a cAMP-independent manner. To investigate the effect of extracellular nucleotides on CFTR activity we have used CHO cells stably transfected with CFTR, a heterologous system usually used to assess CFTR function in overexpression conditions (Chang et al., 1998). Previously we have shown that ATP or the Ca^{2+} ionophore A23187 failed to stimulate ^{125}I efflux in CHO-BQl cells (Chappe et al., 1998), showing the absence of a Ca^{2+}-dependent Cl^- permeability and indicating that CHO-BQ1 cells could constitute a simplistic model for assessing modulation of CFTR functions by extracellular nucleotides.

Extracellular ATP Inhibits CFTR Activity by Acting on the P2Y2 Receptor

The finding that ATP and UTP produced equivalent inhibition of FSK-stimulated CFTR activity led us to hypothesize that ATP and UTP might modulate CFTR activity via P2Y2 and/or P2Y4 receptors, since they are both known to be equally activated by these two nucleotides (Rice et al., 1995; Chen et al., 1996; Bogdanov et al., 1998; Suarez-Huerta et al., 2001). Consistently we have detected, using molecular cloning, the presence of P2Y2 but not P2Y4 transcripts in CHO-BQ1 cells. The deduced amino-acid sequences of the hamster P2Y2 receptor shared 98% and 96% of identity with mouse and rat P2Y2 receptor homologues, respectively. This strongly supports a potential role for the P2Y2 receptor in CFTR inhibition.

It is noteworthy that ADP and adenosine failed to inhibit CFTR activity, ruling out the possibility that ATP-induced CFTR inhibition was mediated by ATP hydrolysis products. Thus, our results exclude a role for both ADP receptors such as P2Y1, P2Y12 and the recently cloned P2Y13 receptors (Ralevic & Burnstock, 1998; Communi et al., 2001; Hollopeter et al., 2001), and adenosine receptors. Likewise, the P2X7 receptor, which is the sole P2X receptor subtype to be expressed in CHO-K1 cells (Michel et al., 1998), was not involved in ATP-induced CFTR inhibition. Indeed, the P2X7 receptor has been shown to be insensitive to UTP (Michel et al., 1998), and treatment with KN-62, used to inhibit the P2X7 receptor, failed to alter the ATP-induced responses. Also, the fact that ATP- and UTP-induced Ca^{2+} responses cross-desensitized suggests that both agonists act on a common P2Y receptor. This therefore excludes the involvement of the P2Y11 receptor since it is insensitive to UTP (Communi et al., 1997; Zambon et al., 2001). We also found that UDP failed to promote a rise in $[Ca^{2+}]_i$ in CHO-BQ1 cells, indicating that the $G_{q/11}$ protein-coupled P2Y6 receptor, most potently activated by UDP (Communi et al., 1996), was not functionally detectable in CHO-BQ1 cells. Taken together, our data demonstrate that ATP and UTP act on a common receptor that we identified as the P2Y2 receptor.

Our experiments reveal that the P2Y2 receptor stimulation induced a biphasic $[Ca^{2+}]_i$ rise in CHO-BQ1 cells due to Ca^{2+} release from internal stores followed by capacitative Ca^{2+} entry possibly through

store-operated plasma membrane channels, typical of the PLC pathway. To identify the G proteins involved, we used PTX treatment, which is commonly used to investigate the G protein coupling selectivity of the P2Y2 receptor (Murthy & Makhlouf, 1998; Janssens et al., 1999). Using this approach, we showed that PTX had no significant effect on ATP/UTP-induced Ca^{2+} responses, indicating that P2Y2 receptors promoted $[Ca^{2+}]_i$ rise by coupling to PTX-insensitive G_q-type G proteins, in agreement with previous data showing P2Y2 receptor coupling to phospholipase C via $G_{q/11}$ but not $G_{i/o}$ proteins in CHO cells (Strassheim & Williams, 2000). Although previous studies have suggested that $G\alpha_i$ proteins may inhibit CFTR activity, possibly by regulating vesicle trafficking and exocytosis mechanisms (Schwiebert et al., 1992; 1994; Schreiber et al., 2001), our data rather suggest that CFTR inhibition by P2Y2 receptors involves the $G\alpha_{q/11}$ protein pathway.

Role of Ca^{2+} in CFTR Activity

Classically, two major effector pathways are activated by G_q protein-coupled receptors: stimulation of PKC and mobilization of Ca^{2+}. Because stimulation of PKC is often related to the stimulation of CFTR rather than to its inhibition (Jia et al., 1997; Chappe et al., 2003), we tested whether ATP-induced CFTR inhibition could be mimicked by a rise in $[Ca^{2+}]_i$. A23187, a Ca^{2+} ionophore, failed to replicate the inhibitory effect of ATP and UTP on CFTR, indicating that an increase in Ca^{2+} was not sufficient to mimic P2Y2 receptor-mediated CFTR inhibition. However, A23187- and ATP/UTP-evoked $[Ca^{2+}]_i$ responses had significantly different time courses. Indeed, ATP/UTP promoted a biphasic Ca^{2+} response with a pronounced plateau, whereas A23187 induced a monophasic $[Ca^{2+}]_i$ rise that was lacking the plateau phase. This perhaps suggests that the plateau phase, which was mediated by capacitative Ca^{2+} entry, may play a key role in mediating CFTR inhibition.

Keeping with this, we noticed that Ca^{2+} signals in response to low nucleotide concentrations ($< 3\mu M$) were asynchronous and detected in only a subset of cells ($\sim$30%). By contrast, higher nucleotide concentrations produced synchronized and biphasic Ca^{2+} responses that were detected in all the cells. Very similar synchronized Ca^{2+} oscillations have been previously described in response to other agonists (Røttingen & Iversen, 2000). Importantly, high concentrations of UTP and ATP (25 μM and 35 μM, respectively) were required to obtain robust inhibitory effects on CFTR, a range of concentrations that consistently evoked biphasic and synchronized Ca^{2+} responses. This reinforces the idea of a causal link between these particular Ca^{2+} responses and CFTR inhibition.

cAMP is not Involved in ATP-induced CFTR Inhibition

A possible alternative mechanism in mediating CFTR inhibition is by decreasing the intracellular cAMP level. We found that, on the contrary, ATP, induced a slight rise in cAMP, but not large enough to activate CFTR. Moreover, while ATP applied simultaneously with IBMX provoked a larger increase in cAMP, it decreased IBMX-induced CFTR activation, further indicating that ATP-induced CFTR inhibition was not due to a decrease in cAMP level. The slight cAMP increase induced by ATP could result either from phosphodiesterase (PDE) inhibition or adenylyl cyclase (*AC*) activation. CHO cells express both the AC isoform VII (ACVII), which is stimulated by PKC and G-protein $\beta\gamma$ subunit, and the ACVI which is inhibited by Ca^{2+} (Sunahara, Dessauer & Gilman, 1996; Varga et al., 1998), and also the PDE1 subtype activated by PKC pathway (Spence et al., 1997). Hence, and somehow speculative, the slight increase in cAMP induced by ATP may result from the concordant activation of ACVII and PDE1. This could explain the potentiating effect of the PDE inhibitor IBMX on the ATP-induced cAMP increase. Collectively, our data indicate that the inhibitory effects of ATP on CFTR are downstream to the cAMP production, possibly on PKA or CFTR.

Effect of CFTR Activation on $[Ca^{2+}]_i$

It has been suggested that CFTR activation can produce ATP release and thereby activates P2Y2 receptors (Urbach & Harvey, 1999), leading to a putative autocrine inhibition of CFTR. We found that neither FSK nor IBMX produced a detectable rise in $[Ca^{2+}]_i$, suggesting that CFTR activation failed to promote an ATP release large enough to activate P2Y2 receptors. Furthermore, we observed that neither FSK nor IBMX treatment altered the ATP-induced Ca^{2+} response, indicating that cAMP increase and CFTR activation did not desensitize P2Y2 receptors. Our data are in line with previous studies that demonstrated that ATP release in CHO-BQ1 cells was independent of CFTR channels (Grygorczyk et al., 1996; Grygorczyk & Hanrahan, 1997).

In conclusion, the present work shows for the first time that, in a heterologous model, CFTR activity is downregulated by P2Y2 receptors in a cAMP-independent manner. At this juncture, we cannot distinguish whether the P2Y2 receptor inhibits CFTR by a mechanism involving Ca^{2+} signal, by intermediate messengers or by more direct interaction with the receptor-G protein complex. Further investigations are therefore needed to identify the molecular mechanisms involved in this ATP-induced CFTR inhibition. Such a nucleotide-induced negative regulation of

CFTR activity may represent a putative therapeutic treatment in secretory diarrheas in decreasing over-activated CFTR channels. Furthermore, although we demonstrate that nucleotide could downregulate CFTR activity, our data are not at all in contradiction with the possible use of UTP as substitutive therapeutic agent in CF disease, which in most cases does not exhibit functional CFTR channels, as well as in chronic bronchitis where CFTR could be functional. Indeed, it has been shown that CaCC is upregulated in CF (Tarran et al., 2002) suggesting that CaCC could be downregulated by CFTR (Wei et al., 1999). Therefore, our results suggest that UTP may have dual effects on Cl^- secretion in inhibiting CFTR and thereby in potentiating CaCC activation to promote hydration of airway secretions.

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