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# Purinoceptor activation of chloride transport in cystic fibrosis and CFTR-transfected pancreatic cell lines

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**1** The regulation of chloride efflux from cystic fibrosis pancreatic adenocarcinoma cells (CFPAC-1) and wild-type CFTR-transfected CFPAC-1 cells (TPAC) was compared.

**2** Forskolin (10 $\mu$M) stimulated chloride efflux from the corrected TPAC cells but not from CFPAC-1 cells. Chloride efflux from both cell types was activated by thapsigargin (0.5 $\mu$M).

**3** The nucleotides ATP and UTP and the non-hydrolyzable ATP analogue, adenosine 5′-O-(3-thio) triphosphate (ATP$\gamma$S), stimulated chloride efflux from both cell types. None of the other $P_2$ purinoceptor agonists investigated elicited a response. The order of potency was ATP$\geqslant$UTP$\geqslant$ATP$\gamma$S.

**4** Adenosine (10–100 $\mu$M) activated choride efflux from the TPAC but not the CFPAC cell line with no increase in intracellular cyclic AMP. Small but statistically significant inhibitions of the adenosine-(50 $\mu$M)-stimulated increase in chloride efflux were elicited by the $A_1$ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX, 100 nM) and the $A_2$ receptor antagonist 3,7-dimethyl-1-propylargylxanthine (DMPX, 10 $\mu$M). The $A_{2A}$ receptor antagonist 8-(3-chlorostyryl)caffeine (CSC, 100 nM) had no significant effect.

**5** These results provide evidence for the regulation of chloride efflux by $P2Y_2$ purinoceptors in genetically-corrected and CF pancreatic cell lines. Studies with adenosine receptor antagonists indicate some possible involvement of $A_1$ and $A_2$ (but not $A_{2A}$) receptors in the adenosine stimulation of chloride efflux, but the relatively small effects of the inhibitors coupled with lack of increase in cyclic AMP and a response only in the CFTR-transfected cells also suggests a possible direct effect of adenosine on CFTR.

**Keywords:** Cystic fibrosis; chloride transport; purinoceptors; adenosine; purine nucleotide; pyrimidine nucleotide; CFPAC-1 cells; CFTR-transfected CFPAC-1 cells

## Introduction

Cystic fibrosis (CF) is a fatal genetic disease caused by a defect in cyclic AMP dependent $Cl^-$ transport in exocrine epithelia (Quinton, 1990). It has been shown that mutation of the CF gene leads to absence, dysregulation or dysfunction of the $Cl^-$ channel encoded by the gene, the cystic fibrosis transmembrane conductance regulator (CFTR) (Welsh & Smith, 1993). CFTR is thought to play an important role in the secretion of a $HCO_3^-$-rich fluid by pancreatic duct cells, required to flush digestive enzymes produced by the pancreatic acini towards the duodenum where it also neutralizes the acid chyme entering from the stomach. The apically located CFTR secretes $Cl^-$ into the ductal lumen where it is reabsorbed by the $Cl^-/HCO_3^-$ exchanger, resulting in net secretion of $HCO_3^-$ (Marino *et al*., 1991; Argent & Gray, 1997). In CF, ductal $HCO_3^-$ and fluid secretions are reduced, leading to concentration and precipitation of the enzymes which ultimately results in duct blockage and destruction of the gland (Durie & Forstner, 1989).

In contrast to the loss of cyclic AMP dependent $Cl^-$ transport, activation of $Cl^-$ channels by alternative mechanisms, such as membrane depolarization and increases in cytosolic $Ca^{2+}$, is not defective in CF cells. The effects of secretagogues which activate $Ca^{2+}$ dependent $Cl^-$ transport were demonstrated to be retained in airway epithelial cells from CF patients (Widdicombe 1986; Boucher *et al*., 1989) and in pancreatic duct cells from transgenic CF mice (Gray *et al*., 1994). Novel therapies targeting the abnormal epithelial ion transport are expected to redress the imbalance in fluid and electrolyte transport apparent in CF epithelia. Activation of alternative $Cl^-$ channels such as the $Ca^{2+}$ dependent $Cl^-$ channels provide a target for the development of therapeutic strategies in treating this disease (Alton *et al*., 1992; Stableforth, 1994).

Purinergic agonists have been demonstrated to elicit a variety of biological responses by interaction with specific purinoceptors in a number of different cell types, adenosine and ATP activating $P_1$ and $P_2$ receptors respectively. Originally $P_1$ receptors were divided into two subtypes, $A_1$ receptors which inhibited adenylate cyclase and $A_2$ receptors which stimulated adenylate cyclase (Van Calker *et al*., 1979). It is now known that at least four subtypes of adenosine receptor exist, $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ which are distinguishable on the basis of agonist and antagonist potency orders. Furthermore, recent research demonstrates that the $P_1$ receptors may mediate their effects through phospholipase C activation and elevation of intracellular $Ca^{2+}$ (for review see Muller & Stein, 1996). The $P_2$ purinoceptors are also divided into two main subgroups: P2X, a family of ligand-gated ion channels; and P2Y, which belong to the G protein-coupled receptor superfamily and elicit their responses through phospholipase C signalling mechanisms (Burnstock & Kennedy, 1985; Abbracchio & Burnstock, 1994). As with the $P_1$ receptors, $P_2$ receptors may be identified on the basis of agonist potency orders (O'Connor *et al*., 1991; Fredholm *et al*., 1997).

A number of research groups have investigated the regulation of $Cl^-$ secretion by purinoceptors. Chao *et al*, (1994) and Stutts *et al*. (1994) described activation of airway ion transport mediated by an $A_2$ receptor utilizing a $Ca^{2+}$

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signalling pathway. Subsequently, Stutts *et al.* (1995) demonstrated that ATP stimulated a cyclic AMP dependent $Cl^-$ transport in $T_{84}$ cells, which was abolished by $\alpha\beta$MeADP. This group concluded that ATP metabolism lead to the activation of an $A_2$ receptor which mediated this effect *via* cyclic AMP dependent activation of the CFTR. $A_1$ receptors have also been reported to mediate increases in $Cl^-$ secretion from airway epithelial cells by a mechanism involving intracellular $Ca^{2+}$ mobilization (Rugolo *et al.*, 1993).

$P_2$ receptors have also been shown to stimulate $Cl^-$ transport in epithelial tissues. Mason *et al.* (1991) demonstrated increased ion transport and elevations of intracellular $Ca^{2+}$ in response to ATP in both CF and normal nasal epithelia, and postulated that the two effects might have a regulatory relationship. Knowles *et al.* (1991) reported increased $Cl^-$ secretion across CF airway epithelia in response to extracellular ATP and UTP, but adenosine and uridine were ineffective in mediating this response. Clarke & Boucher (1992) demonstrated that in normal and CF nasal epithelia, basolaterally applied ATP stimulated $Cl^-$ secretion by an indirect mechanism involving activation of $K^+$ channels, while application of ATP to the apical membrane directly activated $Cl^-$ transport. Stutts *et al.* (1992) observed $Cl^-$ channel activation in both CF and normal airway tissue following application of ATP, while Nilius *et al.* (1995) and Zegarra-Moran *et al.* (1995) demonstrated ATP regulated $Ca^{2+}$ dependent ion transport in renal epithelial cells.

Purinergic agonists may therefore be of use in the treatments of CF. Consequently, we have investigated the ability of these agents to activate $Cl^-$ efflux from relevant human pancreatic epithelial cell lines: CFPAC-1 cells which exhibit the basic CF defect (Schoumacher *et al.*, 1990) and wild-type CFTR-transfected CFPAC-1 cells (TPAC cells) (Drumm *et al.*, 1990) in which the CF defect has been genetically corrected. These cells were used to compare regulation of $^{36}Cl$ efflux in CF and corrected cells and to identify the receptors by which the response to purinergic stimulation is mediated. A preliminary account of some of these data has been published previously in abstract form (O'Reilly & Ryan, 1995).

## Methods

### *Cell culture*

CFPAC-1 cells (passage 16–32) were routinely cultured on plastic culture flasks and maintained in Iscove's modified Dulbecco's medium supplemented with 10% foetal calf serum and 500 iu $ml^{-1}$ penicillin/streptomycin. TPAC cells (passage 27–34) were cultured in the same medium with the addition of 0.4 mg $ml^{-1}$ geneticin. Cells were grown at 37°C in an atmosphere of 95% $O_2$/5% $CO_2$, medium was changed every 2 days. For radioisotope efflux experiments, cells were grown to confluency in Falcon 6-well plates. For cyclic AMP assays, cells were grown to confluency in 24-well plates.

### *Measurement of chloride efflux*

$H^{36}Cl$ was neutralized with equimolar KOH to generate $K^{36}Cl$. Confluent cells were washed with uptake buffer consisting of 135 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, 10 mM Glucose, 20 mM HEPES, pH 7.4 with NaOH. Cells were loaded with 2 ml uptake buffer containing 0.5 $\mu$Ci $ml^{-1}$ $K^{36}Cl$ for 2 h at 37°C. Cells were transferred to a 25°C water bath and incubated for 15 min before being washed in efflux buffer containing 135 mM Na gluconate, 3 mM K gluconate, 1.5 mM Mg gluconate, 1.3 mM Ca gluconate, 10 mM Glucose, 20 mM HEPES, pH 7.4 with NaOH. Cells were then bathed with 2 ml efflux buffer at 25°C for the duration of the experiment. At appropriate time points after initiation of the time course, duplicate 0.5 ml samples of the bathing buffer were removed to scintillation vials. One ml of efflux buffer was added to the solution bathing the cells to replace the volume removed. After the last time point, the remaining efflux buffer was also transferred to a scintillation vial. Controls were prepared by treating blank wells in an identical manner. 0.9 ml of 2% sodium dodecyl sulphate was added to each well, followed by 2.1 ml $H_2O$, in order to solubilize the cells. Duplicate 250 $\mu$l samples of the lysed cell suspension were retained for protein analysis according to the method of Lowry *et al.* (1951) the remainder was transferred to a scintillation vial. Ten ml Ecoscint A scintillation fluid was added to each vial and all samples were assayed for radioisotope content using an LKB 1217 Wallac Rackbeta liquid scintillation counter. Total radioactivity was determined by counting 10 $\mu$l samples of loading buffer. Efflux was expressed as a fraction of total $Cl^-$ present in the cells when efflux began (nmoles $Cl^-$ effluxed/total nmoles $Cl^-$ present when efflux was initiated), time zero was designated as the time drugs were added. The apparent efflux rate constants ($k_a$) were calculated for efflux from CFPAC-1 and TPAC cells under control conditions and in cells treated with forskolin, thapsigargin and a combination of forskolin and thapsigargin. Efflux rates were calculated for each time point using the equation

$$k_a \quad Q \quad t \quad 1 \quad P_o \quad Q$$

where $P_o$ is the initial amount of radioisotope in the cell and Q is the amount of isotope detected in the fluid bathing the cells.

### *Measurement of cyclic AMP levels*

Firstly, cyclic AMP binding protein was prepared from bovine adrenal glands. The adrenal glands were obtained fresh from a local abattoir and placed in ice-cold buffer containing 50 mM Tris and 4 mM EDTA (pH 7.4 at 25°C). Each gland was cut open through the medullary region, which was subsequently removed with a forceps. The cortices were isolated from the peritoneal membranes and surrounding tissue and homogenized in Tris-EDTA buffer. The homogenate was filtered through a muslin sheet and the resultant filtrate was centrifuged at 20,000 *g* for 20 min at 4°C. The supernatant was pipetted off and stored in 1 ml aliquots at −20°C. Confluent cells were incubated with 1 ml fresh medium containing 200 mM IBMX for 35 min at 37°C. Drug treatment followed for 13 min at 37°C. Seventy-five $\mu$l 0.2 M HCl was added to each well and the cells were incubated for 30 min at 4°C. Eighty-six $\mu$l 0.2 M NaOH was then added to each well, cells were removed from the bottom of the wells by scaping and the mixture was triturated to form an even cell suspension. Controls were prepared by treating blank wells in an identical manner. Duplicate 40 $\mu$l aliquots were retained for protein analysis and duplicate 50 $\mu$l aliquots were transferred to test tubes for measurement of cyclic AMP. Fifty $\mu$l $^3H$-cyclic AMP was added to both cell suspension aliquots and cyclic AMP standards. The cyclic AMP binding protein was defrosted and diluted 1/10 with Tris-EDTA buffer. The mixture was hand homogenized and 200 $\mu$l was added to the samples and standards. Following a 4 h incubation at 4°C, the contents of each tube were filtered through Whatman GF/C filters using a Brandel 24-well cell harvester. The filters were transferred to scintillation vials and 10 ml scintillation fluid was added to each. Counting was

performed as described previously and cyclic AMP levels were calculated by comparison to a standard curve. Results were expressed as pmoles cyclic AMP per mg protein.

### *Statistical analysis*

Data are presented as mean ± standard error of the mean (s.e.mean). Significance of differences between mean values of two groups was tested by Student's unpaired *t*-test. Alternatively, for multiple comparisons between control and treatment groups, data were analysed for statistical significance using one way analysis of variance (ANOVA), followed by Dunnett's post test. A probability of $P \leqslant 0.05$ was considered statistically significant. The fraction of $^{36}Cl$ effluxed at the 2 min time point for each concentration of agonists shown was used to calculate $EC_{50}$ values using INPLOT™ software. Maximum response was found with 100 $\mu$M ATP and experiments with 500 $\mu$M ATP (results not shown) indicated similar values for fraction chloride effluxed at 100 and 500 $\mu$M ATP. The rank order of potency of the agonists was assessed at the concentrations used.

### *Materials*

CFPAC-1 cells were purchased from the European Collection of Animal Cell Cultures. TPAC cells were the generous gift of R. Frizzell, University of Alabama, Birmingham, U.S.A. $H^{36}Cl$ (354 $\mu$Ci mmol$^{-1}$) and $^3$H-cyclic AMP (28 Ci mmol$^{-1}$) were obtained from Amersham. Forskolin; ATP; UTP; IBMX; cAMP; adenosine; adenosine 5′-O-(3-thio) triphosphate (ATP$\gamma$S); $\alpha,\beta$-methylene ATP ($\alpha\beta$MeATP); $\beta,\gamma$-methylene ATP ($\beta\gamma$MeATP); ADP; adenosine 5′-O-(2-thio) diphosphate (ADP$\beta$S); adenosine 5′-($\alpha,\beta$-methylene) diphosphate ($\alpha\beta$ MeADP); and geneticin were from Sigma (Poole, Dorset, U.K.). Thapsigargin; 2-Methylthio ATP (2MeSATP); 8-cyclopentyl-1,3-dipropylxanthine (CPX); 8-(3-chlorostyryl)caffeine (CSC) and 3,7-dimethyl-1-propargylxanthine (DMPX) were purchased from Research Biochemicals International (Natick, MA, U.S.A.). All cell culture solutions were obtained from Gibco (Scotland). All other chemicals were Analar.

## Results

Initial experiments were undertaken to demonstrate the differences in transepithelial ion transport between the two cell types under investigation. $Cl^-$ efflux was examined in both cell types in the presence and absence of forskolin (Figure 1). Basal efflux of $^{36}Cl$ from TPAC cells was significantly higher than that observed from CFPAC-1 cells, $P \leqslant 0.005$. Addition of forskolin (10 $\mu$M) to the buffer bathing the TPAC cells led to a marked and statistically significant increase in $Cl^-$ efflux from these cells compared to control, $P \leqslant 0.01$. After 10 min the fraction of total $Cl^-$ effluxed from untreated cells was $0.43 \pm 0.05$, while that from forskolin-treated cells was $0.69 \pm 0.02$. No such increase was observed in the CFPAC-1 cells where fractional efflux from untreated and treated cells was $0.31 \pm 0.04$ and $0.30 \pm 0.01$ respectively.

Thapsigargin (0.5 $\mu$M) brought about large statistically significant increases in $Cl^-$ efflux from both TPAC ($P \leqslant 0.005$) and CFPAC-1 ($P \leqslant 0.05$) cells throughout the time course observed (Figure 2). These increases were greater in the TPAC cells than in the CFPAC-1 cells. The fraction of $Cl^-$ effluxed was raised to $0.76 \pm 0.02$ in TPAC cells and to $0.51 \pm 0.02$ in the CFPAC-1 cells, after 10 min of efflux in each cell type.

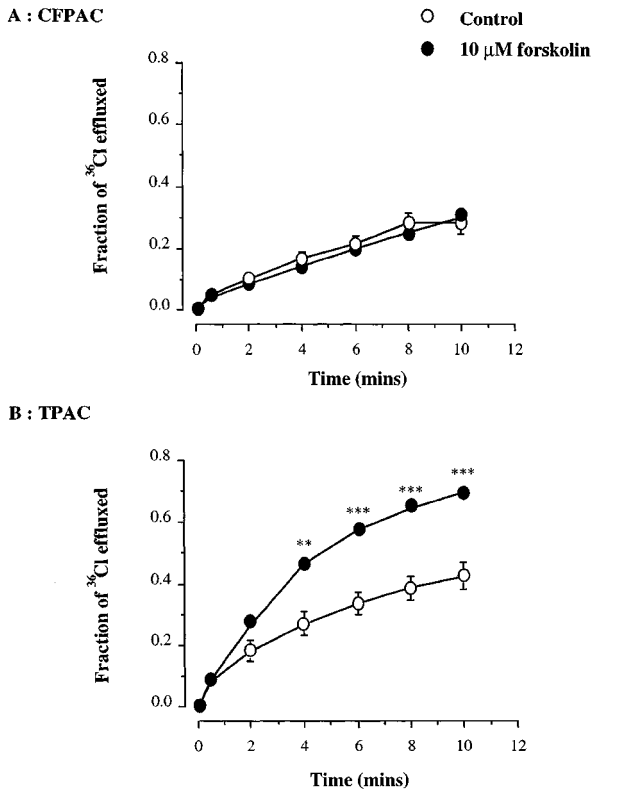


**Figure 1** $^{36}Cl$ efflux from CFPAC-1 (A) and TPAC cells (B), measured in the presence and absence of 10 $\mu$M forskolin. No response to forskolin stimulation was observed in the CFPAC-1 cells, however a significant increase in efflux from TPAC cells was elicited by forskolin, $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.005$, Student's *t*-test. Results were expressed as the fraction of total $Cl^-$ effluxed from the cells. Values are means ± s.e.mean, $n = 3-6$.

The rate of $^{36}Cl$ efflux from CFPAC-1 and TPAC cells was calculated (Figure 3). In both cell types the initial $k_a$ calculated at 0.5 min was higher than those determined at later time points under the different experimental conditions. Statistical comparison of the $k_a$s from CFPAC-1 cells indicated that forskolin did not significantly alter the rate of efflux when compared to control cells, at any of the time points examined. In contrast, efflux from CFPAC-1 cells treated with either thapsigargin or a combination of forskolin and thapsigargin proceeded with a significantly higher $k_a$ than control cells, $P \leqslant 0.01$ for both treatment groups for the duration of the time course. The $k_a$s calculated for efflux from TPAC cells indicated that individually both forskolin and thapsigargin significantly increased the rate of efflux from these cells when compared to control cells, $P \leqslant 0.05$ and $P \leqslant 0.01$ respectively. The $k_a$ calculated for TPAC cells treated with a combination of forskolin and thapsigargin were significantly higher than those determined for either drug alone at the 0.5 and 2 min time points, $P \leqslant 0.05$ for each group. At the later time points, significance was detected only in comparison with the untreated TPAC cells ($P \leqslant 0.01$), no significant differences between cells treated with either forskolin or thapsigargin and a combination of these two agents was detected.

A range of purinergic agonists were examined for their ability to increase $^{36}Cl$ efflux from both cell types. Addition of ATP to the solution bathing the cells caused a significant increase in fractional efflux from both cell types when analysed with ANOVA and Dunnett's post test (Figure 4). The increase in $Cl^-$ efflux was statistically significant ($P \leqslant 0.01$) at 10 and

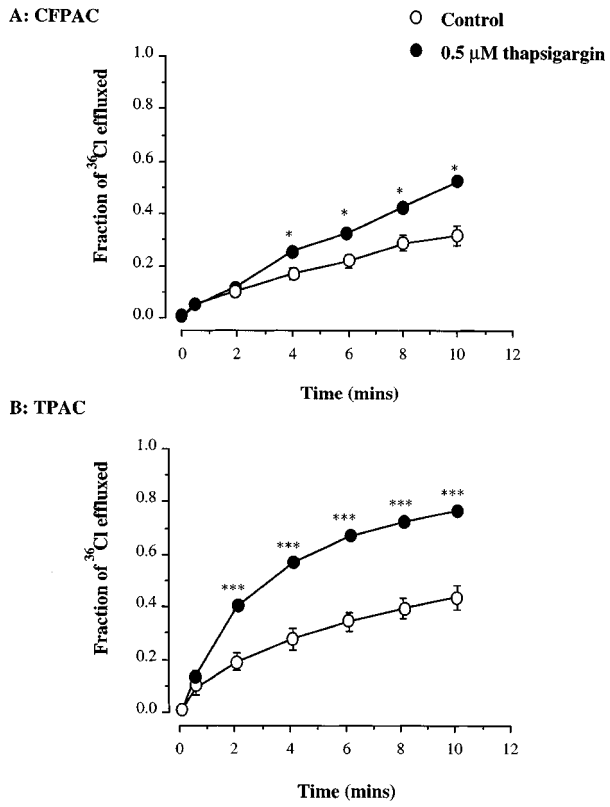


**Figure 2** $^{36}Cl$ efflux from CFPAC-1 (A) and TPAC cells (B), measured in the presence and absence of 0.5 $\mu$M thapsigargin. Significant increases in efflux from both CFPAC-1 and TPAC cells were stimulated by thapsigargin. Results were expressed as the fraction of total $Cl^-$ effluxed from the cells. Values are means $\pm$ s.e.mean, $n=3-6$. $^*P\leqslant0.05$, $^{**}P\leqslant0.01$, $^{***}P\leqslant0.005$, Student's $t$ test.

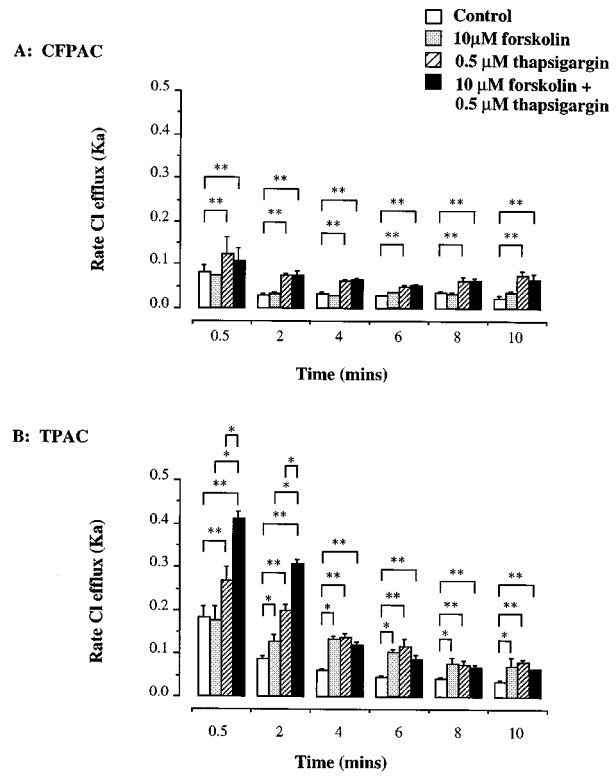


**Figure 3** Efflux rate constants ($k_a$) calculated for CFPAC-1 (A) and TPAC cells (B) under the following conditions: control, 10 $\mu$M forskolin, 0.5 $\mu$M thapsigargin, 10 $\mu$M forskolin and 0.5 $\mu$M thapsigargin. Values are means $\pm$ s.e.mean, $n=3-6$. $^*P\leqslant0.05$, $^{**}P\leqslant0.01$, $^{***}P\leqslant0.005$, ANOVA and Dunnetts post test.

100 $\mu$M doses of ATP for both cell types. $EC_{50}$ values were determined for the response to ATP, this was calculated to be 5.6 $\mu$M in CFPAC-1 cells and 1.7 $\mu$M in TPAC cells. Treatment of the monolayers with UTP also caused an increase in $^{36}Cl$ efflux to be observed in CFPAC-1 and TPAC cells (Figure 5). The response observed in CFPAC-1 cells was significant at doses of UTP equal to 1, 10 and 100 $\mu$M ($P\leqslant0.01$). The $EC_{50}$ value for this response was estimated at 5.8 $\mu$M. In TPAC cells, significantly increased $Cl^-$ efflux occurred at doses of 10 $\mu$M ($P\leqslant0.05$) and 100 $\mu$M ($P\leqslant0.01$) UTP. The $EC_{50}$ value for this response was found to be 6.2 $\mu$M. ATP$\gamma$S was also examined for its ability to elicit increases in $Cl^-$ transport from both cell types (Figure 6). Significant increases in $Cl^-$ efflux were obtained in both CFPAC-1 and TPAC cells when treated with 100 $\mu$M ATP$\gamma$S ($P\leqslant0.05$). These increases were lower than the responses observed when ATP and UTP were applied to the cells. The $EC_{50}$ values determined for the effects of ATP$\gamma$S on efflux from CFPAC-1 and TPAC cells were 7.4 $\mu$M and 4.4 $\mu$M respectively. The effects of other purinergic agonists upon $Cl^-$ efflux were also examined. $\alpha\beta$MeATP, $\beta\gamma$MeATP, 2MeSATP, ADP and ADP$\beta$S did not significantly affect the amount of $Cl^-$ effluxed from either cell type (results not shown). The rank order of potency for stimulation of $^{36}Cl$ efflux was therefore observed as ATP $\approx$ UTP $\geqslant$ ATP$\gamma$S $>>$ $\alpha\beta$MeATP, $\beta\gamma$MeATP, 2MeSATP, ADP and ADP$\beta$S for CFPAC-1 cells and ATP $\geqslant$ UTP $\geqslant$ ATP$\gamma$S $>>$ $\alpha\beta$MeATP, $\beta\gamma$MeATP, 2MeSATP, ADP and ADP$\beta$S for TPAC cells.

Adenosine was found to have no significant effect upon $^{36}Cl$ efflux from CFPAC-1 cells (Figure 7). In contrast, TPAC cells treated with adenosine demonstrated a dose-dependent increase in $^{36}Cl$ efflux, significant elevations in secretion being observed at concentrations of 10, 50 and 100 $\mu$M ($P\leqslant0.01$), with an $EC_{50}$ value of 8.1 $\mu$M. Ten $\mu$M adenosine failed to stimulate an increase in the intracellular concentration of cyclic AMP in either cell type. In fact, in both cell lines there was a tendency, though not statistically significant, for a decrease in cyclic AMP levels following treatment with 10 $\mu$M adenosine as follows: CFPAC cells-basal 49.2 $\pm$ 17.7 compared to 25.0 $\pm$ 5.0 in adenosine-treated cells, TPAC cells-basal 57.3 $\pm$ 14.2 compared to 30.7 $\pm$ 13.2 in adenosine-treated cells – all values are pmoles/mg protein and values given are means $\pm$ s.e.mean. In control experiments, forskolin (10 $\mu$M) increased the cyclic AMP levels to 1423 $\pm$ 384 in CFPAC cells and to 1243 $\pm$ 174 in TPAC cells indicating similar responses in cyclic AMP to this compound in both cell types. $P_1$ purinoceptor antagonists were used in attempts to identify the receptors involved in the response to 50 $\mu$M adenosine by TPAC cells. The $A_1$ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX), (100 nM), elicited a small (19% at 6 min) but statistically significant inhibition of adenosine-stimulated $^{36}Cl$ efflux ($P\leqslant0.01$) but was without effect upon basal efflux (Figure 8A). Ten $\mu$M 3,7-dimethyl-l-propylargylxanthine (DMPX), an $A_2$ receptor antagonist, was also observed to partially inhibit adenosine-stimulated $^{36}Cl$ efflux ($P\leqslant0.01$) and its effects were somewhat greater (28% at 6 min) than those of CPX (Figure 8B). Increasing the ratio of antagonists to adenosine did not produce any greater inhibition of $^{36}Cl$ efflux (data not shown). The $A_{2A}$ specific receptor antagonist 8-(3-chlorostyryl)caffeine (CSC), (100 nM), did not significantly inhibit either basal or adenosine-stimulated chloride efflux from these cells (Figure 8C).

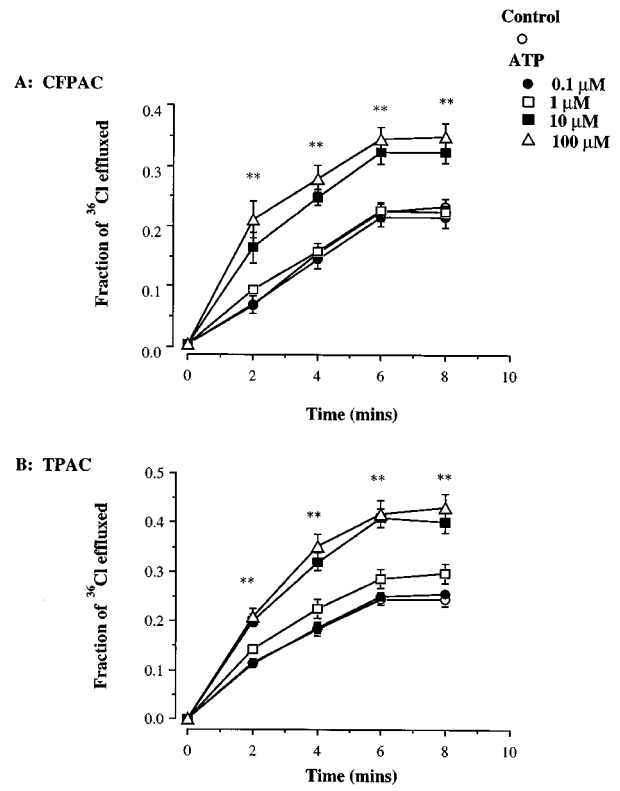


**Figure 4** $^{36}$Cl efflux from CFPAC-1 (A) and TPAC cells (B), measured in the presence of increasing concentrations of ATP; 0 $\mu$M, 0.1 $\mu$M, 1 $\mu$M, 10 $\mu$M and 100 $\mu$M. Significant increases in efflux from both cell types were elicited by 10 $\mu$M ($P \leqslant 0.01$) and 100 $\mu$M ($P \leqslant 0.01$) ATP. $EC_{50}$ values were calculated to be 5.6 $\mu$M in CFPAC-1 cells and 1.7 $\mu$M in TPAC cells. Results were expressed as the fraction of total $Cl^-$ effluxed from the cells. Values are means$\pm$s.e.mean, $n=3-6$. $^{**}P \leqslant 0.01$, 10 $\mu$M and 100 $\mu$M ATP compared to control (0 $\mu$M ATP), ANOVA.

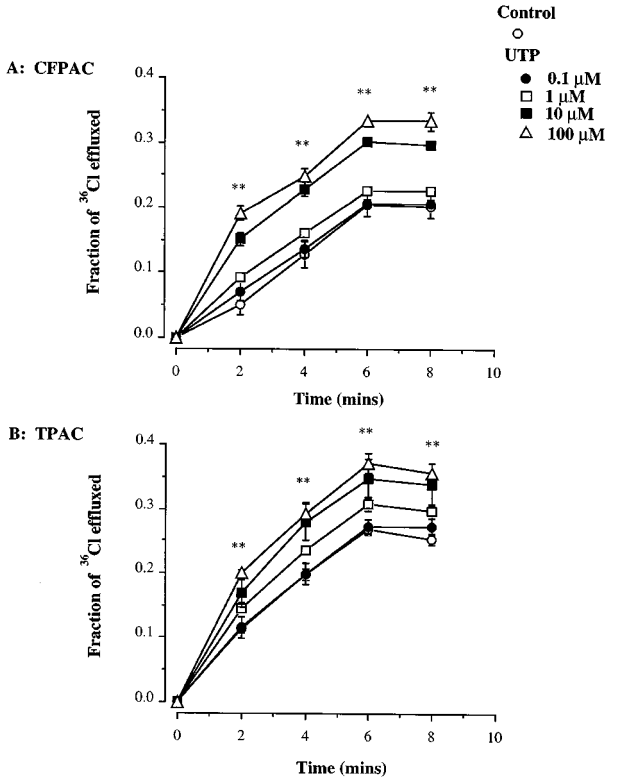


**Figure 5** $^{36}$Cl efflux from CFPAC-1 (A) and TPAC cells (B), measured in the presence of increasing concentrations of UTP; 0 $\mu$M, 0.1 $\mu$M, 1 $\mu$M, 10 $\mu$M and 100 $\mu$M. Significant increases in efflux from CFPAC-1 cells were elicited by 1, 10 and 100 $\mu$M UTP ($P \leqslant 0.01$), an $EC_{50}$ value of 5.8 $\mu$M was calculated. $^{36}$Cl efflux from TPAC cells was also stimulated by UTP, significance was detected at 10 and 100 $\mu$M doses ($P \leqslant 0.01$). The $EC_{50}$ value for this response was determined to be 6.2 $\mu$M. Results were expressed as the fraction of total $Cl^-$ effluxed from the cells. Values are means$\pm$s.e.mean, $n=3-5$. $^{**}P \leqslant 0.01$, 10 $\mu$M and 100 $\mu$M UTP compared to control (0 $\mu$M UTP), ANOVA.

The effect of the 5′-ectonucleotidase inhibitor $\alpha\beta$MeADP (100 $\mu$M) upon responses to 100 $\mu$M ATP was evaluated in both CFPAC-1 and TPAC cells (Figure 9). In neither cell type was control or ATP-stimulated $^{36}$Cl efflux from cells altered by incubation with $\alpha\beta$MeADP.

## Discussion

Expression of the CF defect by CFPAC-1 cells was demonstrated by the reduced basal efflux observed from these cells when compared to efflux from TPAC cells. Furthermore, forskolin, which stimulates adenylate cyclase synthesis of cyclic AMP, thus increasing intracellular levels of this second messenger, is known to activate $Cl^-$ transport in a number of different cell types: human airway epithelia (Haws *et al.*, 1994); Kunzelmann *et al.*, 1994), human and rat colonic epithelia (Vaandrager *et al.*, 1991; Fuller *et al.*, 1994), amphibian retinal pigment cells (Hughes & Segawa, 1993), porcine thyroid cells (Armstrong *et al.*, 1992). In this study, forskolin activated $Cl^-$ efflux from the TPAC cells but failed to produce any response from the CFPAC-1 cells (Figure 1). This illustrates the basic defect which causes CF, a lack of cyclic AMP mediated $Cl^-$ channels in CF cells. This is in agreement with the findings of Schoumacher *et al.* (1990) and Drumm *et al.* (1990), which demonstrated that while CFPAC-1 cells failed to respond to forskolin stimulation, retroviral transfection of CFPAC-1 cells with wild-type CFTR restored cyclic AMP regulated anion transport in these cells.

Efflux from CFPAC-1 and TPAC cells was measured in $Cl^-$ free buffer, in which $Cl^-$ was replaced by gluconate which is expected to prevent activation of anion exchange mechanisms (Mastrocola *et al.*, 1991). This was in order to eliminate the possibility of carrier mediated transport, proceeding through an anion exchange mechanism located at the apical membrane, contributing to basal levels of $^{36}$Cl efflux, for example $^{36}C <\!\!-\!\!> Cl^-$ exchange. Under these conditions, differences in $Cl^-$ transporting capabilities of the two cell lines were observed. Both cell types investigated demonstrated a higher rate of efflux at the beginning of the time course when compared to efflux during the later time points. This may be due to the presence of isotope in the extracellular matrix prior to the commencement of efflux or alternatively the replacement of $Cl^-$ with gluconate in the efflux buffer may have created a gradient for $^{36}$Cl efflux. However, as the decrease in efflux rate was more marked in stimulated cells than untreated cells, it may also reflect down regulation of the receptors or ion channels involved in mediation of the response to the agents employed in these experiments.

An inhibitor of the endoplasmic reticulum calcium ATPase, thapsigargin discharges intracellular $Ca^{2+}$ stores, thus causing an increase in the concentration of intracellular

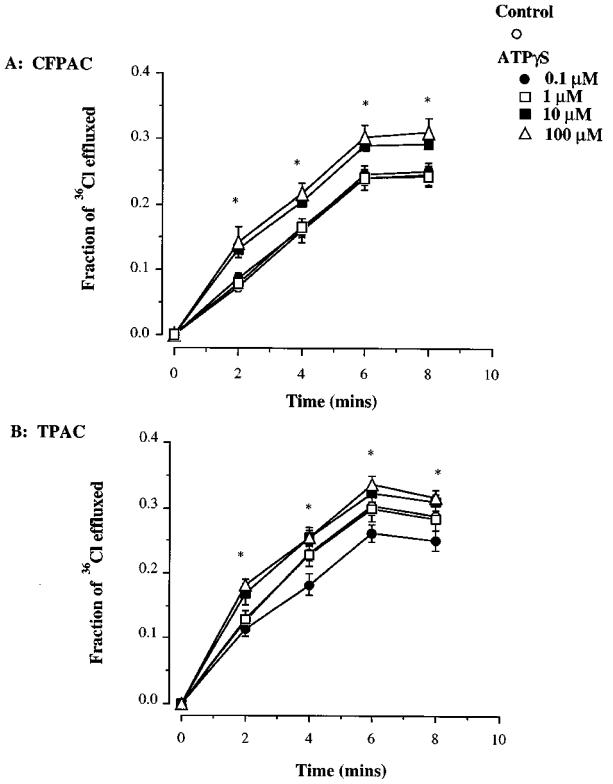


**Figure 6** $^{36}Cl$ efflux from CFPAC-1 (A) and TPAC cells (B), measured in the presence of increasing concentrations of ATP$\gamma$S; 0 $\mu$M, 0.1 $\mu$M, 1 $\mu$M, 10 $\mu$M and 100 $\mu$M. Significant increases in efflux from both cell types were elicited by 100 $\mu$M ATP$\gamma$S ($P \leqslant 0.05$) when compared to control. $EC_{50}$ values were calculated to be 7.4 $\mu$M in CFPAC-1 cells and 4.4 $\mu$M in TPAC cells. Results were expressed as the fraction of total $Cl^-$ effluxed from the cells. Values are means $\pm$ s.e.mean, $n = 5$. *$P \leqslant 0.05$, 100 $\mu$M ATP$\gamma$S compared to control (0 $\mu$M ATP$\gamma$S), ANOVA.

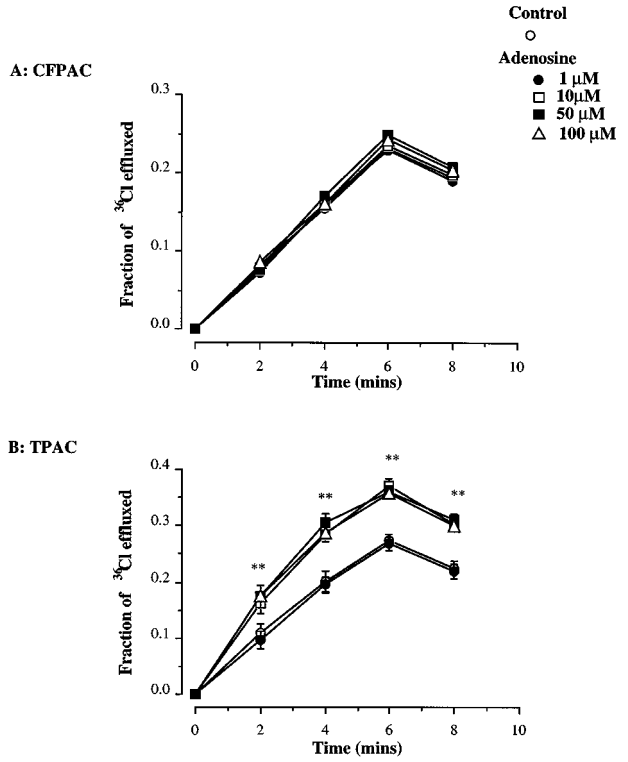


**Figure 7** $^{36}Cl$ efflux from CFPAC-1 (A) and TPAC cells (B), measured in the presence of increasing concentrations of adenosine; 0 $\mu$M, 1 $\mu$M, 10 $\mu$M, 50 $\mu$M, 100 $\mu$M. Significant increases in efflux were detected from TPAC cells at concentrations of 10, 50 and 100 $\mu$M, with an $EC_{50}$ value of 8.1 $\mu$M. Results were expressed as the fraction of total $Cl^-$ effluxed from the cells. Values are means $\pm$ s.e.mean, $n = 3$. **$P \leqslant 0.01$, 10 $\mu$M, 50 $\mu$M and 100 $\mu$M adenosine compared to control (0 $\mu$M adenosine), ANOVA.

$Ca^{2+}$ (Thastrup *et al*., 1990; Pickles & Cuthbert, 1992). This leads to activation of $Ca^{2+}$ dependent processes within the cell, including activation of ion channels (Pickles & Cuthbert, 1991; Kachinthorn *et al*., 1993). Both CFPAC-1 and TPAC cells responded to thapsigargin with significant increases in $^{36}Cl$ efflux. These findings are in accordance with previous reports that increased anion transport has been observed in CF tissues in response to increases in cytosolic $Ca^{2+}$ (Boucher *et al*., 1989; Willumsen *et al*., 1989). Boucher *et al*. (1989) demonstrated preservation of this $Cl^-$ secretory mechanism in human CF airway. This has also been observed in the mouse model of CF by Leung *et al*. (1995). Interestingly, the response in TPAC cells was greater than that observed in CFPAC-1 cells, despite the fact that $Ca^{2+}$ dependent $Cl^-$ transporting processes are not thought to be affected by the CF defect. TPAC cells displayed a faster rate of efflux initially, which eventually plateaued towards the end of the time course. In contrast, thapsigargin stimulated efflux from CFPAC-1 cells proceeded in a linear fashion and had not reached a maximum by the end of the time course. The effect of thapsigargin stimulation upon the rate of efflux in the presence of forskolin was determined and TPAC cells showed additivity of the two responses, where combination of the two agonists lead to significantly greater responses than elicited from either agonist alone (Figure 3). Synergism has been reported between cyclic AMP and $Ca^{2+}$ mediated cellular processes and this may be responsible for the apparent differences observed between the cells (Cartwright *et al*., 1985; Vajanaphanich *et al*., 1995). It is possible that increases in intracellular $Ca^{2+}$ lead to the activation of $Ca^{2+}$ dependent $K^+$ channels, thus providing an increased driving force for efflux of $^{36}Cl$ through cyclic AMP activated $Cl^-$ channels (Roch *et al*., 1995). Alternatively, increases in cytosolic $Ca^{2+}$ may also lead to $Ca^{2+}$ dependent phosphorylation of the CFTR which would lead to CFTR activation and hence to the greater rate of efflux observed (Piciotto *et al*., 1992; Berger *et al*., 1993). These results demonstrate maintenance of $Ca^{2+}$ dependent pathways for $Cl^-$ secretion in CF cells, and illustrate the possibility of overcoming the CF defect by activation of cyclic AMP independent $Cl^-$ conductances in CF affected cells. Thus therapy aimed at re-establishing normal ion transport in CF patients may find a basis in activation of $Ca^{2+}$ dependent $Cl^-$ channels.

Initial experiments with ATP showed that both CFPAC-1 and TPAC cells expressed $P_2$ receptor-mediated $Cl^-$ secretory pathways. These findings are in agreement with those reported by Galietta *et al*. (1994) and Chan *et al*. (1996) who demonstrated ATP and UTP activated $Cl^-$ secretion from CFPAC-1 cells suggesting the presence of a $P2Y_2$ receptor. However, none of the work previously reported investigated the effects of these agonists upon $^{36}Cl$ efflux from TPAC cells, nor the possibility of P2X or $P2Y_1$ receptor involvement in the response to ATP. Therefore a range of nucleotide analogues, known to act at specific $P_2$ purinoceptors, were tested for their ability to stimulate $^{36}Cl$ efflux. Of the nucleotide agonists tested in this series of experiments, only ATP, UTP and ATP$\gamma$S were

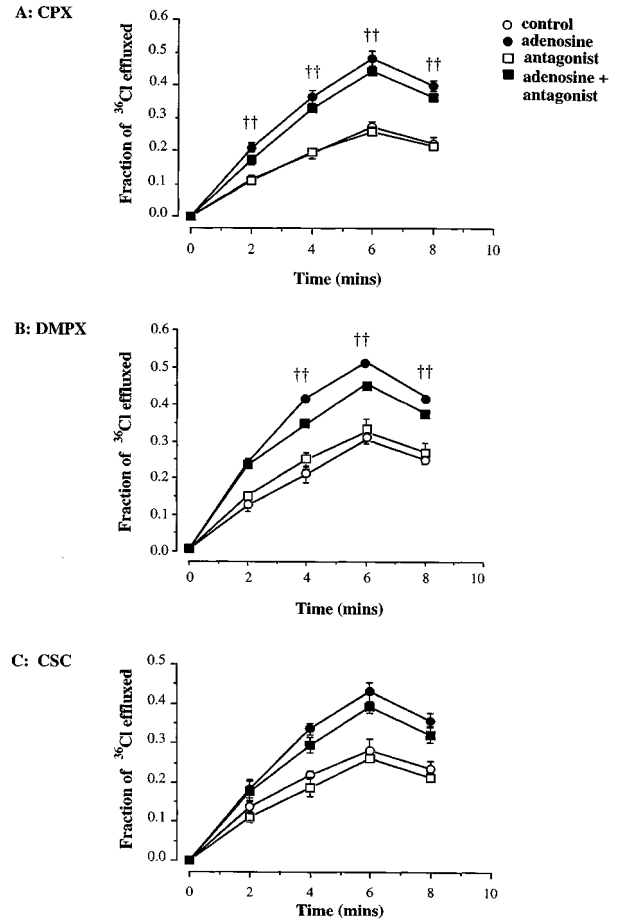


**Figure 8** The effect of $P_1$ receptor antagonists upon basal and 50 $\mu$M adenosine stimulated $^{36}Cl$ efflux from TPAC cells. (A) The $A_1$ receptor antagonist CPX (100 nM) had no effect upon basal efflux from TPAC cells but produced a small but statistically significant inhibition of adenosine-stimulated $Cl^-$ secretion (††$P \leqslant 0.01$). (B) DMPX (10 $\mu$M), an $A_2$ receptor antagonist, elicited a somewhat greater inhibition of adenosine-stimulated $^{36}Cl$ efflux (††$P \leqslant 0.01$) but did not reduce basal efflux. (C) The $A_{2A}$ antagonist CSC (100 nM) had no statistically significant effect upon either basal or adenosine-stimulated $^{36}Cl$ efflux. Results were expressed as the fraction of total $Cl^-$ effluxed from the cells for the following groups: control, adenosine, antagonist and adenosine + antagonist. Values are means $\pm$ s.e.mean, $n = 3$. Statistical analysis by ANOVA and Dunnett's post test. ††$P \leqslant 0.01$ antagonist plus adenosine compared to adenosine-stimulated $^{36}Cl$ efflux.

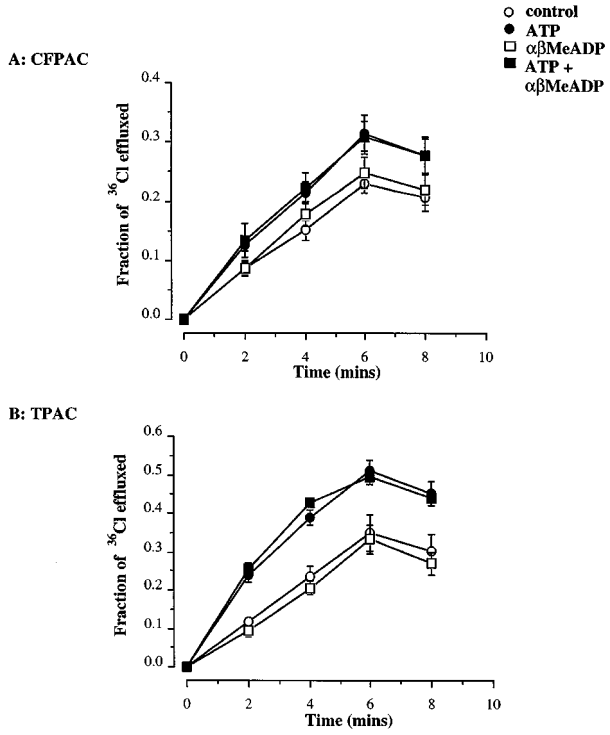


**Figure 9** The effect of $\alpha\beta$MeADP upon control and ATP stimulated $^{36}Cl$ efflux from CFPAC-1 and TPAC cells. Neither control nor ATP activated $^{36}Cl$ efflux from CFPAC-1 or TPAC cells were affected by $\alpha\beta$MeADP. Results were expressed as the fraction of total $Cl^-$ effluxed from the cells for the following groups: control, ATP, $\alpha\beta$MeADP and ATP + $\alpha\beta$MeADP. Values are means $\pm$ s.e.mean, $n = 6$. Statistical analysis by ANOVA and Dunnett's post test.

shown to significantly increase efflux from epithelial cells. Based on the classification of purinoceptors by O'Connor *et al.* (1991) these data suggest the presence of a $P_{2U}$ purinoceptor (renamed $P2Y_2$) on the membrane of pancreatic epithelial cells. The data are also in accordance with the observed potency orders of the recombinant $P2Y_2$ receptors described by Lustig *et al.* (1993), Webb *et al.* (1993) and Parr *et al.* (1994). Our conclusions were, in part, supported by calculation of $EC_{50}$ values for each of the agonists investigated. The results indicated that each of the agonists (ATP, UTP and ATP$\gamma$S) exhibited similar levels of potency in both cell types, with the exception of ATP which had an $EC_{50}$ value of 1.7 $\mu$M in TPAC cells, considerably lower than that determined for its actions in CFPAC-1 cells (5.6 $\mu$M). This finding suggests that ATP is therefore considerably more potent in stimulating secretion from TPAC cells than from CFPAC-1 cells. As the TPAC cells express functional CFTR, unlike the CFPAC-1 cells, the increased potency of ATP in these cells could be explained by activation of this $Cl^-$ channel. This might occur either (i) through production of adenosine by metabolism of ATP and subsequent activation of $A_2$ adenosine receptors resulting in an increase in intracellular cyclic AMP or (ii) ATP or adenosine-induced increases in intracellular $Ca^{2+}$ concentrations might lead to protein kinase C activation of the CFTR or (iii) direct activation of CFTR by ATP or adenosine.

Adenosine was demonstrated to stimulate $^{36}Cl$ efflux from the TPAC cells but was without effect in the CFPAC-1 cells, consistent with activation of CFTR by adenosine. However, this response did not appear to be mediated by cyclic AMP as no significant increases in cyclic AMP concentrations within either cell type were observed during exposure to 10 $\mu$M adenosine. Results from experiments using $P_1$ receptor antagonists, suggested that both $A_1$ and $A_2$ (but not $A_{2A}$) receptors contributed, at least, to some extent in adenosine-stimulated $^{36}Cl$ efflux. The observation that $A_2$ receptors elicit cyclic AMP-independent activation of $Cl^-$ secretion is in agreement with research performed on airway epithelial cells (Stutts *et al.*, 1994; Chao *et al.*, 1994). However the involvement of $A_1$ receptors in the mediation of the response to adenosine is more controversial. Eidelman *et al.* (1992) and Guay-Broder *et al.* (1995) demonstrated that $A_1$ receptor antagonists activated $^{36}Cl$ efflux from CFPAC-1 cells and a CF tracheal cell line but not from genetically corrected cells. This response was shown to be independent of $A_1$ receptor inhibition (Jacobsen *et al.*, 1995). McCoy *et al.* (1995) reported that an $A_1$ receptor antagonist did not affect basal ion transport from either CF or normal airway epithelial cells. However, pretreatment of cells with the antagonist increased

$Cl^-$ channel activation in response to 8-(4-chlorophenylthio)-cyclic AMP (CPTcAMP, a membrane permeable cyclic AMP analogue) in normal cells and surprisingly caused channel activation in response to CPTcAMP in CF cells. In conflict with those reported findings, Rugolo *et al.* (1993) demonstrated that $A_1$ receptor agonists stimulated $Cl^-$ channel activation in both CF and normal airway epithelial cells. In our studies, adenosine stimulated $^{36}Cl$ efflux only from TPAC cells and not CFPAC cells. This also raises the possibility of direct effects of adenosine on CFTR. The CFPAC cells carry the Δ508 mutation which results in misprocessing of the CFTR resulting in a quantitative deficit of CFTR in the plasma membrane. Pollard and colleagues (Pollard, 1997; Cohen *et al.*, 1997) have provided evidence for direct binding of xanthines and adenosine to CFTR. This binding can result in activation of CFTR. Adenosine may therefore in the present studies have produced enhanced $^{36}Cl$ efflux in TPAC cells by direct activation of CFTR. The lack of effect of adenosine in CFPAC cells may reflect the deficit of CFTR at the cell membrane in these cells. The fact that no increase in cyclic AMP was detected in TPAC cells after adenosine treatment also suggests the unlikelihood of a major involvement of $A_2$ receptors in the stimulation of $^{36}Cl$ efflux by adenosine.

In the present studies, it was also considered whether metabolism of ATP could be generating products which were activating ion secretion from TPAC cells but not from CFPAC-1 cells. The process of ATP metabolism proceeds along the following pathway: ATP→ADP→AMP→adenosine. A number of different enzymes are responsible for mediating the different stages of breakdown of ATP. $\alpha\beta$MeADP is an inhibitor of ATP metabolism, acting upon ecto-5′-nucleotidase which is responsible for hydrolysis of AMP, thus generating adenosine (Ziganshin *et al.*, 1994; Meghji *et al.*, 1995; Meghji & Burnstock, 1995). To examine the hypothesis that adenosine was contributing to the observed increase in $^{36}Cl$ efflux in response to ATP, the experiments were repeated in the presence and absence of $\alpha\beta$MeADP. No significant differences in the response to ATP were noted when cells were exposed to $\alpha\beta$MeADP, therefore leading to the conclusion that metabolism of ATP to adenosine was not contributing to stimulation of $^{36}Cl$ efflux by ATP in these cell lines. Also in support of this argument is the finding that ADP did not have the same effect as ATP but would be expected to be broken down to adenosine.

In conclusion, these cells provide a very useful model for the comparison of ion transporting properties of CF and non-CF cell types. The basic biochemical defect associated with CF was clearly demonstrated, but the presence of alternative mechanisms by which $Cl^-$ secretion may be activated indicates that these cell types may be used in the development of a pharmacological strategy to treat the disease. Further work is required to investigate the mechanisms by which ATP and adenosine stimulate chloride transport in these cells and, in particular, the possible role for direct activation of CFTR. The observation that $P2Y_2$ receptors are capable of eliciting activation of $Cl^-$ secretion demonstrates their potential in providing a method of overcoming the basic ion transport defect in CF.

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