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Actions of adenosine A_1 and A_2 receptor antagonists on CFTR antibody-inhibited β-adrenergic mucin secretion response

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1 The cystic fibrosis gene protein, the cystic fibrosis transmembrane conductance regulator (CFTR) acts as a chloride channel and is a key regulator of mucin secretion. The mechanism by which 3-isobutyl-1-methylxanthine (IBMX) corrects the defect in CFTR mediated β-adrenergic stimulation of mucin secretion has not been determined. The present study has investigated the actions of adenosine A_1 and A_2 receptor antagonists to determine whether ability to stimulate mucin secretion correlates with correction of CFTR antibody inhibited β-adrenergic response and whether excessive cyclic AMP rise is required.

2 CFTR antibodies were introduced into living rat submandibular acini by hypotonic swelling. Following recovery, mucin secretion in response to isoproterenol was measured.

3 The adenosine A_1 receptor antagonist, 8 cyclopentyltheophylline (CPT) was a less potent stimulator of mucin secretion than was the A_2 receptor antagonist dimethylpropargylxanthine (DMPX). A concentration of CPT close to the K_i for A_1 receptor antagonism (10 nM) did not stimulate mucin secretion.

4 DMPX, although a potent stimulator of mucin secretion, did not correct CFTR antibody inhibited mucin secretion.

5 CPT corrected defective CFTR antibody inhibited mucin secretion at a high (1 mM) concentration, suggesting a mechanism other than adenosine receptor antagonism.

6 DMPX potentiated the isoproterenol induced cyclic AMP rise, whereas CPT did not.

7 Correction of the defective CFTR mucin secretion response did not correlate with ability to stimulate mucin secretion and did not require potentiation of β-adrenergic induced increases in cyclic AMP. This affords real promise for the development of a selective drug treatment for cystic fibrosis.

Keywords: Adenosine receptor antagonists; mucin secretion; CFTR; submandibular glands

Introduction

Cystic fibrosis (CF), a common lethal inherited disease of Caucasians, is characterized by an altered composition of epithelial cell mucous secretions (McPherson & Dormer, 1991; 1992). It is not known how alteration in activity of the CF gene protein, CFTR, which has cyclic AMP-dependent Cl^- channel activity (Riordan, 1993) causes the severe respiratory disease (Dormer & McPherson, 1994). Nevertheless, CFTR has been shown to be a key regulator of mucin secretion (McPherson *et al*., 1986; 1988; 1991; Bradbury *et al*., 1992; Lloyd Mills *et al*., 1992; Kuver *et al*., 1994; McPherson & Dormer, 1994). Thus, an antibody raised against a synthetic peptide from the first nucleotide binding domain of CFTR inhibited β-adrenergic stimulated mucin secretion, when introduced into living rat submandibular cells by hypotonic swelling (Lloyd Mills *et al*., 1992). The β-adrenergic receptors controlling mucin secretion in rat submandibular glands are predominantly β_1-receptors (Bradbury *et al*., 1989). The acinar cells of rat submandibular glands secrete mainly fluid and high molecular weight mucins (Simson *et al*., 1978; Quissell & Barzen, 1980). Cells containing CFTR antibody showed the same phenotype as submandibular gland cells from CF individuals (McPherson *et al*., 1986; 1988) and CF mice (Lloyd Mills *et al*., 1995). They thus provide a good model for investigating correction of the CFTR defect. An antibody raised against an adjacent peptide sequence in CFTR inhibited cyclic AMP-dependent Cl^- transport (Chan *et al*., 1992). In addition, cells transfected with CFTR showed increased cyclic AMP dependent endocytosis and exocytosis (Bradbury *et al*., 1992) and mucin secretion (Kuver *et al*., 1994).

Pharmacological correction of the CFTR-mediated mucin secretion defect has been demonstrated (McPherson *et al*., 1986; 1988). Thus, defective β-adrenergic stimulation of mucin secretion in CF cells and in CFTR antibody containing cells was corrected to the same degree by the non-selective compound IBMX (McPherson *et al*., 1986; 1988; Lloyd Mills *et al*., 1992), which acts as an adenosine receptor antagonist, a cyclic nucleotide phosphodiesterase inhibitor and protein phosphatase inhibitor (Dormer & McPherson, 1994; Becq *et al*., 1994; Guay-Broder *et al*., 1995). IBMX was shown to activate CFTR dependent Cl^- transport in cells expressing ΔF508-CFTR (Drumm *et al*., 1991; Haws *et al*., 1996). The present study has investigated the actions of adenosine A_1 and A_2 receptor antagonists on CFTR antibody inhibited submandibular cells to directly determine whether ability to stimulate mucin secretion correlates with correction of the defective CFTR-mediated β-adrenergic response and whether potentiation of the β-agonist induced cyclic AMP rise is required. Some of the data has been published in preliminary form (Pereira *et al*., 1998).

Methods

Production of anti-peptide CFTR antibodies

A peptide consisting of 14 amino acids (524–537), residing in the first nucleotide binding domain (NBD) region, of CFTR

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(Riordan *et al*., 1989), was synthesized and coupled to KLH (10 mg peptide/8 mg KLH) by Cambridge Research Biochemicals Ltd, Northwich Cheshire, U.K. The peptide sequence (CQLEEDISKFAEKD) was searched for in the Swissprot database (BLASTP and FASTA3_T, 1997) and only human and bovine CFTR were found to contain a perfect match. Rat CFTR (CQLQEDITKFAEQD) has 11 identical and three conserved amino acids in this 14 amino acid sequence; the mouse sequence is identical to rat, but with an additional change (Q at position 5) from rat and human. Antisera were prepared as described, by injection of conjugates (100–200 μg ml^{-1}), emulsified in Freunds adjuvant, intradermally into rabbits (Lloyd Mills *et al*., 1992; Pereira *et al*., 1991). The antisera were affinity-purified using KLH or peptide coupled to CH-Sepharose 4B (Pharmacia), with elution of antibody fractions in phosphate buffered saline, pH 7 or 0.1 M glycine-HCl respectively. IgG fractions were prepared by precipitation with 24% Na_2SO_4 for 2 h at room temperature. The IgG content was estimated using the BioRad protein assay kit.

Immunoprecipitation/phosphorylation of murine CFTR

CFTR-transfected and mock-transfected CHO cells (Landsell *et al*., 1998) were grown and maintained in Hams 92/F-12 nutrient medium supplemented with 10% foetal calf serum, 600 μg ml^{-1} neomycin, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Confluent cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 10 mM iodoacetate, 1 mM PMSF, 10 μg ml^{-1} each of chymostatin, pepstatin A, antipain, aprotinin and leupeptin) for 30 min on ice. Nuclear/cell debris was removed by micocentrifugation (13,000 × *g*) for 15 min at 4°C. The supernatant was incubated with affinity-purified CFTR antibody for 90 min at 4°C and antibody-CFTR complex precipitated with Pansorbin (10% suspension of Staphylococcus aureus cells prewashed in RIPA buffer). The precipitate was washed, resuspended in phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mg ml^{-1} BSA) and phosphorylated *in vitro* (60 min at 37°C) using the catalytic subunit of protein kinase A (75 nM) and 10 μCi of [γ^{32}P]-ATP. Phosphorylation was terminated by addition of RIPA and after several washes the immune complex dissociated by solubilization in electrophoresis sample buffer (0.125 M Tris-HCl, pH 6.8, 5% SDS, 25% sucrose, 5% mercaptoethanol) for 15 min at 37°C. Samples were subjected to electrophoresis on 7.5% polyacrylamide gels followed by Coomassie-Blue staining. Gels were then dried and autoradiographed overnight using Hyperfilm-MP (Amersham).

Results (Figure 1) show that the antibody raised against the human NBD peptide cross reacted with mouse CFTR, expressed in CHO cells. Cross reaction with a C terminal CFTR antibody (Lloyd Mills *et al*., 1992) is also shown for comparison. Our previous results have shown that both the NBD and the C-terminal CFTR antibody recognize CFTR in native rat and mouse submandibular and pancreatic tissues (Lloyd Mills *et al*., 1992).

Isolation of rat submandibular acini, incorporation of antibodies into intact acini and measurement of mucin secretion

Procedures were carried out by minor modification of those previously described (McPherson & Dormer, 1984; Lloyd Mills *et al*., 1991; 1992). Briefly, acini were pulse-chase labelled

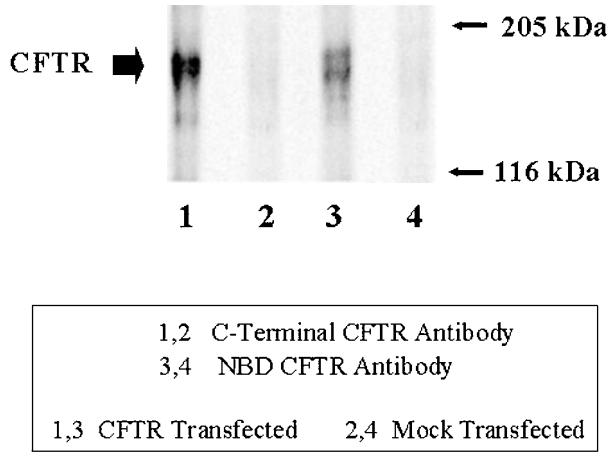

Figure 1 Recognition of mouse CFTR by CFTR antibodies. CFTR- and mock-transfected CHO cells were extracted, immunoprecipitated and phosphorylated as described in the Methods. The results, which are representative of three experiments, show autoradiographs following SDS polyacrylamide gel electrophoresis of samples immunoprecipitated with C terminal CFTR antibody or NBD CFTR antibody. Position of molecular weight markers are shown. CFTR runs with a molecular weight of 180–190 kDa.

with [^{3}H]-glucosamine (5 μCi ml^{-1}) and suspended in TES-buffered saline (10 mM TES, pH 7.4 containing (in mM) NaCl 143, KCl 4.7, $MgCl_2$ 1.1, BSA 1 mg ml^{-1}). To 200 μl of acini suspension, 800 μl of either 10 mM TES, pH 7.4 (swollen) or Tes-buffered saline (unswollen), each containing 5 mM ATP and CFTR antibody or non-immune IgG (approximately 1 mg IgG ml^{-1}) was added for 1.5 min at room temperature, followed by washing and resuspension in KHB buffer (see McPherson & Dormer, 1984; Lloyd Mills *et al*., 1992) containing 20 mg ml^{-1} BSA. Following a 15 min recovery incubation at 37°C in KHB buffer, acini were washed and incubated under experimental conditions at 37°C. Isoproterenol and IBMX were dissolved directly into KHB medium at the concentrations used; the adenosine antagonists (DMPX K_i = 11 μM at A_2; 45 μM at A_1 receptors and CPT, K_i = 10.9 nM at A_1, 1440 nM at A_2 receptors; obtained from RBI Research Biochemicals Inc., Natick, U.S.A.) were dissolved initially in dimethylsulphoxide (DMSO) and diluted to give a final concentration of 1% DMSO in the incubations. An equivalent amount of DMSO was added to control incubations. [^{3}H]-glucosamine-labelled mucins, released into the medium at zero time and after 30 min, were acid-precipitated using a combination of 10% trichloroacetic acid (TCA) and 0.5% phosphotungstic acid (PTA). The precipitates were washed three times in TCA/PTA (10%/0.5%) at 4°C and their radioactivity measured as previously described (McPherson & Dormer, 1984; Lloyd Mills *et al*., 1992).

The protein content of cell pellets was determined using the BioRad protein assay kit and mucin release over 30 min expressed as d.p.m./mg protein or as % basal secretion to take account of variation in unstimulated mucin release between experiments.

Lactate dehydrogenase (LDH) release was measured spectrophotometrically, in media and cells (extracted in 56 mM Tris-HCl, pH 7.4 containing 5.6 mM EDTA, 0.02% (v/v) Triton X-100, 0.1% (w/v) soybean trypsin inhibitor), by following the oxidation of NADH in the presence of sodium pyruvate at 340 nm.

Characterization of the secreted products

Acini were pulse chase labelled with D-(+)-[1-^{14}C]-glucosamine and incubated for 60 min in the presence of isoproterenol (10 μM), as described (McPherson & Dormer, 1984). Medium was removed and secreted glycoproteins precipitated with 10% TCA/0.5% PTA as described (McPherson & Dormer, 1984) and analysed as described below:

Gel filtration The TCA/PTA insoluble material was dissolved in 0.1 M Tris-HCl containing 0.1% sodium dodecyl sulphate, 1 mM dithiothreitol and 1 mM EDTA, pH 9.0. The TCA/PTA soluble material was dialyzed overnight against the same buffer. Material (TCA/PTA insoluble and soluble) was applied separately to a Biogel P-200 column, fractionated and radioactivity counted.

Cellulose acetate electrophoresis The TCA/PTA precipitated radioactively-labelled material in 0.01 M Tris-HCl pH 8.0 (2 μl sample applied), was electrophoresed for 4 h on cellulose acetate (Shandon) sheets in 0.2 M calcium acetate pH 7.2 buffer at a current of 6 mA per sheet (Waddington *et al*., 1988). The lanes were cut into sections (approximately 2 mm) and counted for radioactivity by liquid scintillation counting. The amount of radioactivity in the sections was compared with that of TCA/PTA insoluble material subjected to either digestion with chondroitinase ABC in 0.1 M tris/HCl, pH 8.0 (0.5 u ml^{-1}; overnight at 37°C), which specifically digests dermatan sulphate, chondroitin-4-sulphate and chondroitin-6-sulphate, digestion with trypsin (30 μg bovine pancreatic; Sigma Type XIII added to 30 μl of sample for 8 h at 37°C followed by a second addition of 30 μg and incubation overnight at 37°C) or treatment with nitrous acid (18% sodium nitrite in glacial acetic acid for 90 min at room temperature), which specifically oxidises N-sulphated groups in heparin and heparan sulphate (Last *et al*., 1988; Waddington *et al*., 1988) and electrophoresed in the same way. As positive controls, a standard mixture containing 0.05 mg ml^{-1} each of hyaluronic acid, heparan sulphate, dermatan sulphate, chondroitin-4-sulphate and chondroitin-6-sulphate was electrophoresed on cellulose acetate before and after the above treatments, under the conditions described and visualized by Alcian blue staining in 3% acetic acid/0.05 M $MgCl_2$. Chondroitinase ABC digestion and nitrous acid treatment caused complete loss of Alcian blue staining.

Caesium chloride density gradient centrifugation The methods used were based on those described by Thornton *et al*., 1990. The TCA/PTA precipitated radioactively-labelled material was dissolved in 0.5 M NaOH, dialyzed overnight against double distilled water and freeze-dried. The freeze-dried material (approximately 10,000 d.p.m.) or bovine submandibular mucin (50 μg, Sigma) was dissolved in 4 M guanidinium-HCl and solid CsCl added to a starting density of 1.42 g ml^{-1}. Samples were centrifuged at 15°C for 48 h at 100,000 $\times g$ using a swing out rotor. Gradients were fractionated and aliquots (400 μl) of each fraction counted for radioactivity by liquid scintillation counting. Aliquots of fractions (50 μl) were also dot-blotted onto 0.45 μm nitrocellulose membrane and stained using Periodic Acid-Schiff (PAS) as follows: blots were incubated at room temperature in 1% (w/v) periodic acid in 3% (v/v) acetic acid for 30 min, washed with water and immersed in 0.1% (w/v) sodium metabisulphite in 0.01 M HCl for 10 min, followed by 15 min incubation in Schiff's reagent then rinsing and incubation in metabisulphite as before. After a final rinse in water, the membrane was air dried and the intensity of staining in each dot measured by densitometric scanning using the BioRad image analysis system.

Measurement of cyclic AMP content

Aliquots of acini suspensions (0.25 ml) were added to an equal volume of ice cold trichloroacetic acid (20%), extracted and assayed using a specific radioimmunoassay kit for cyclic AMP (Amersham), as previously described (Lloyd Mills *et al*., 1992).

Analysis of data

All values are reported as means ± s.e.mean. Significant differences between two means were determined with Student's *t*-test for unpaired observations. $P < 0.05$ level was considered significant. A one-way analysis of variance (ANOVA) with Bonferroni correction for multiple tests was employed for data shown in Table 3.

Results

Characterization of radioactively labelled secretory products

Figure 2 shows the elution profiles of the secreted ^{14}C-labelled material (TCA/PTA) insoluble and soluble fractions) obtained following pulse chase labelling of acini as described in the Methods. It can be seen that at least 80–90% of the TCA/PTA insoluble labelled material eluted in the void volume of the Biogel P-200 column, having a molecular weight of at least 200 kDa, consistent with the size of the high molecular weight mucins present in rat submandibular glands (Quissell & Barzen, 1980). Figure 2 also shows that there is a negligible amount of high molecular weight material in the TCA/PTA soluble fraction.

Cellulose acetate electrophoresis of the TCA/PTA insoluble material gave a major band stained with Alcian blue near the origin of the sheet (Rf: 0.04), which was similar to that seen on electrophoresis of purified bovine submandibular mucin (Rf: 0.04). Almost all of the radioactivity applied (approximately 800 d.p.m.) was present in this band (99.7, 96.8% of total counts; $n = 2$). Enzyme digestion with chondroitinase ABC or trypsin or nitrous acid treatment, as described in the Methods, yielded recovery of counts in this band of 90.9, 92.1% total, $n = 2$ (chondroitinase), 100.6, 101.1% total, $n = 2$ (trypsin) and

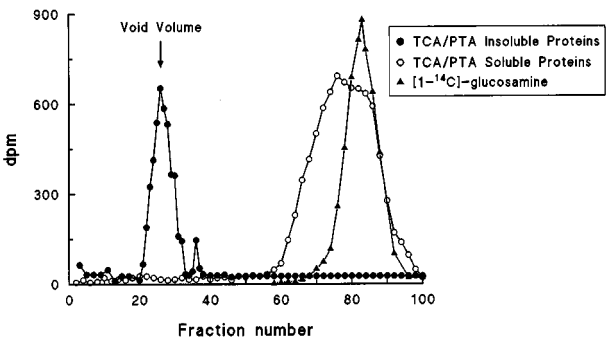

Figure 2 Elution profile of secreted proteins from Biogel P-200. TCA/PTA insoluble and soluble [1-^{14}C]-glucosamine-labelled secreted proteins were applied to a Biogel P-200 column eluted and the radioactivity counted as described in the Methods. [1-^{14}C]-glucosamine was applied separately as a marker. Void volume was calibrated using Dextran blue.

100.7, 97.6% total, $n=2$; (nitrous acid). The results indicate that less than 10% of the TCA/PTA insoluble radioactivity secreted is due to secretion of proteoglycans, with the major component having characteristics of mucin in terms of charge density and resistance to trypsin and nitrous acid. Analysis of the radioactively-labelled secreted proteins on CsCl density gradient centrifugation (Figure 3) showed that they ran as a single peak of density 1.4 g ml^{-1}. This was similar for both basal and stimulated secretions (data from basal secretions not shown). The radioactivity corresponded to the PAS staining of the sample and also to that of purified bovine submandibular mucin. The data shows that the majority of the radioactivity in the TCA/PTA precipitate has characteristics of mucin in both basal and stimulated rat submandibular acinar cell secretions.

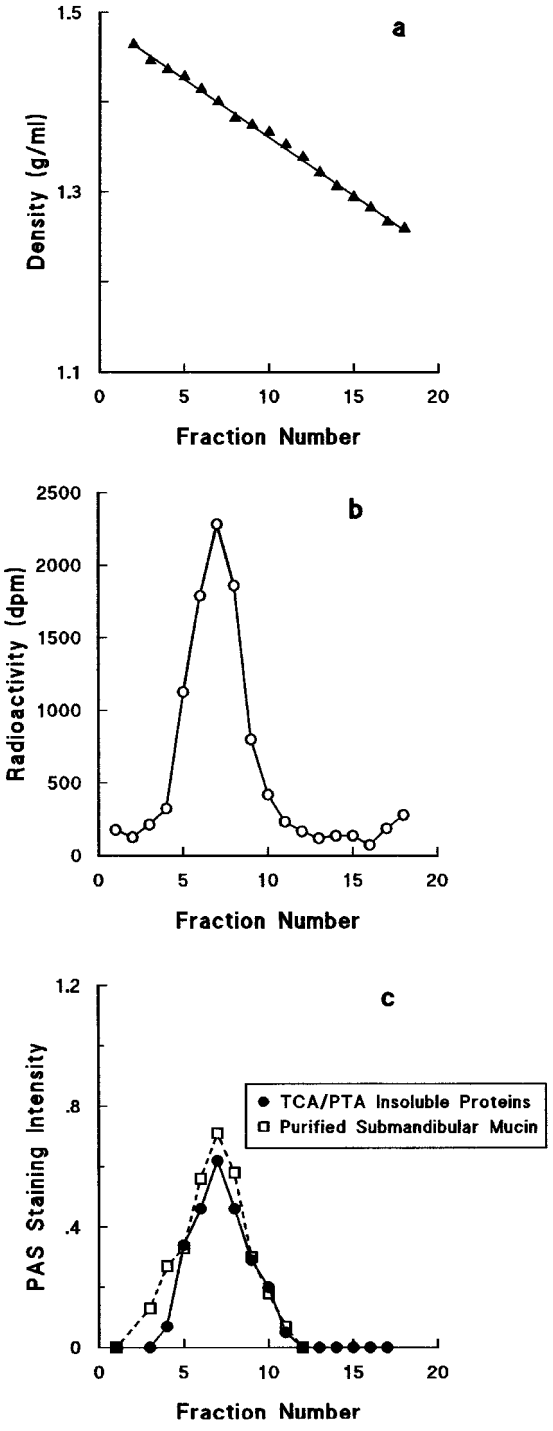

Figure 3 Characterization of TCA/PTA insoluble secreted proteins by CsCl density gradient centrifugation. TCA/PTA precipitated radioactively-labelled material was applied to a CsCl density gradient in 4 M guanidinium-HCl as described in the Methods. Fractions were analysed for density (a), radioactivity (b) and PAS staining (c). The PAS staining profile of purified bovine submandibular mucin, applied to a separate gradient, is shown on the same profile (c) for

Dose-dependence of stimulation of mucin secretion by adenosine receptor antagonists

Figure 4 shows the effects of different concentrations of DMPX and CPT on mucin secretion from rat submandibular acini, isolated as described in the Methods. DMPX was the more effective stimulator, and at 1 mM increased mucin secretion by approximately 4 fold which is similar to that seen with a maximal isoproterenol or IBMX concentration (Table 3). CPT was apparently less potent but significantly increased mucin secretion at a concentration of 1 mM. Doses of CPT close to the K_i for A_1 adenosine receptor antagonism did not stimulate mucin secretion (10 nM CPT; 104.3% basal, $n=4$). The data indicate that the effect of CPT and DMPX in increasing mucin secretion is unlikely to be related to A_1 receptor antagonism, although an A_2 agonist effect cannot be ruled out.

To ensure that responses to agonists are not due to cell lysis, release of the cytoplasmic enzyme, lactate dehydrogenase (LDH) was measured. Table 1 shows that release of LDH is low and is unaffected by CPT or DMPX under conditions of secretion or restoration of defective CFTR mediated response. Thus, effects of agonists on mucin secretion and restoration of secretory response cannot be explained by increases in cell lysis.

Correction of antibody-inhibited defective CFTR function

The actions of selective A_1 and A_2 receptor antagonists on correction of CFTR antibody-inhibited β-adrenergic stimulation of mucin secretion has been investigated. Table 2 shows

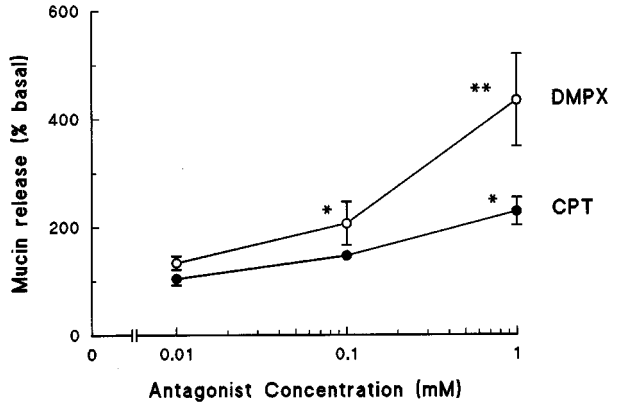

Figure 4 Dose-dependent effect of adenosine receptor antagonists, DMPX and CPT on mucin secretion from rat submandibular acini. Mucin secretion was determined by pulse-chase labelling with [^{3}H]-glucosamine as described in the Methods. Mucin secretion after 30 min was measured in the presence or absence of different concentrations of agonist as shown. Results are means$\pm$s.e.mean for at least four experiments and are expressed as % basal. Basal mucin secretion varied from 389–1117 d.p.m./mg protein. $^*P<0.05$; $^{**}P<0.01$ for difference from basal secretion as assessed by Student's t-test for unpaired data.

Table 1 Effect of agonists on LDH release from rat submandibular acini

	LDH Release	
	Units/mg protein	*% Total cell content*
No addition	1.72±0.24	3.53±0.54
CPT (1 mM)	1.40±0.13	2.87±0.32
DMPX (1 mM)	1.49±0.16	3.05±0.36
Isoproterenol (10 μM)	1.74±0.14	3.56±0.34
Isoproterenol (10 μM)+CPT (1 mM)	1.69±0.39	3.48±0.85
Isoproterenol (10 μM)+DMPX (1 mM)	1.48±0.13	3.03±0.31

Acini were pulse-chase labelled, swollen in the presence of CFTR antibody as described in the Methods and incubated for 30 min in the presence or absence of the agonists shown. LDH release was measured as described in the Methods. Total cell LDH content varied from 47.33–50.67 u mg^{-1} protein. Results are means±s.e.mean for four experiments.

Table 2 Effect of DMPX on isoproterenol-stimulated mucin secretion in rat submandibular acini containing non-immune IgG or CFTR antibody

	Mucin Secretion (% basal)	
	Swollen+NI IgG	*Swollen+CFTR Antibody*
Isoproterenol (10 μM)	228.1±17.5	*155.0±14.5
Isoproterenol+DMPX (1 mM)	245.0±26.6	*167.8±7.4

Rat submandibular acini were pulse-chase labelled with [^{3}H]-glucosamine and swollen in the presence of CFTR antibody or an equivalent amount (approximately 1 mg ml^{-1}) of non-immune IgG as described in the Methods, allowed to recover and then incubated for 30 min under the conditions shown. Mucin secretion was measured as described and expressed as % basal. Results are means±s.e.mean for four experiments. Significance of differences was assessed by Student's *t*-test for unpaired data: $*P<0.05$ for difference from isoproterenol stimulation in cells swollen in non-immune IgG.

that the A_2 adenosine receptor antagonist, DMPX, at a concentration (1 mM) which markedly increases mucin secretion (Figure 4) did not correct defective β-adrenergic stimulation of mucin secretion in CFTR antibody containing cells. As shown previously (Lloyd Mills *et al*., 1992), mucin secretion in response to isoproterenol from cells containing CFTR antibody introduced by hypotonic swelling, was significantly decreased compared to cells swollen in an equivalent amount of non-immune IgG. It can be seen (Table 2) that DMPX had no effect on the normal isoproterenol response in cells containing non-immune IgG or on the decreased isoproterenol response in CFTR antibody-containing cells. Figure 5 shows the dose-dependence of correction of the defective isoproterenol-stimulated mucin secretion in CFTR antibody inhibited cells by the A_1 receptor antagonist CPT. CPT was shown to correct the defective β-adrenergic response at 1 mM. At this concentration, CPT was as effective as 1 mM cpt-cyclic AMP, which was previously shown to restore secretory responsiveness in CFTR antibody-inhibited cells to approximately 75% of that seen in cells containing a similar amount of non-immune IgG (Lloyd Mills *et al*., 1992). CPT at lower concentrations (Figure 5) did not significantly correct the CFTR mucin secretion defect; although a slight increase was seen at a concentration of 0.1 mM CPT. At a concentration close to the K_i for A_1 receptor antagonism it was ineffective, (10 nM CPT; 97.4±8.5% basal, $n=4$). The high concentration of CPT which corrected the CFTR antibody-inhibited mucin secretory response was indicative that a mechanism other than adenosine receptor antagonism was operating.

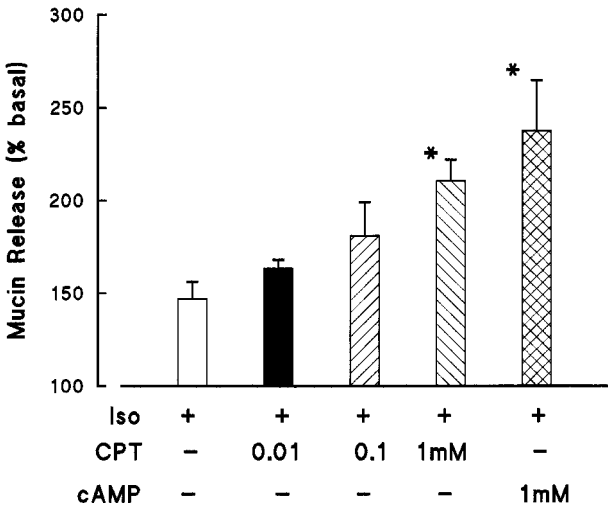

Figure 5 Correction of defective CFTR-mediated isoproterenol-stimulated mucin secretion by the A_1 receptor antagonist, CPT. Rat submandibular acini were pulse-chase labelled with [^{3}H]-glucosamine and swollen in the presence of CFTR antibody as described in the Methods. Mucin secretion, in the presence or absence of agonists as shown, was measured after 30 min. Results are means±s.e.mean for at least four experiments and are expressed as % basal. Basal mucin secretion varied from 476–1730 d.p.m./mg protein. $*P<0.05$ for difference from secretion in the presence of isoproterenol alone as assessed by Student's *t*-test for unpaired data.

Excessive cyclic AMP rise and correction of defective CFTR function

Results showing correction of the CFTR mucin secretion defect by IBMX and cpt-cyclic AMP (Lloyd Mills *et al*., 1992) led to the hypothesis that an excessive increase in cyclic AMP is the mechanism by which defective CFTR function is restored. It was therefore investigated whether correction by the A_1 receptor antagonist, CPT was dependent on potentiation of isoproterenol induced cyclic AMP rise. Table 3 shows the actions of CPT on mucin secretion and cyclic AMP levels in the presence or absence of isoproterenol, compared to those of IBMX. Our previous results have shown that the cyclic

Table 3 Actions of adenosine receptor antagonists on mucin secretion and cyclic AMP levels in rat submandibular acini

	Mucin Secretion (d.p.m./mg protein)	*Cyclic AMP* (pmol mg^{-1} protein)
No addition	794±78 (9)	8.8±2.0 (6)
IBMX (1 mM)	3092±449 (6)	**27.8±4.9 (4)
CPT (1 mM)	*1826±303 (9)	*113.5±20.5 (6)
Iso (10 μM)	3311±324 (11)	199.2±37.8 (6)
Iso (10 μM)+IBMX (1 mM)	3897±1223 (6)	**668.5±69.1 (4)
Iso (10 μM)+CPT (1 mM)	3240±348 (10)	178.8±37.6 (6)

Rat submandibular acini were pulse-chase labelled with [^{3}H]-glucosamine as described in the Methods. Mucin release was measured at 30 min and cyclic AMP at 5 min as described in the Methods, in the presence or absence of agonists. Results are means±s.e.mean for the number of experiments shown in parentheses. A one-way analysis of variance (ANOVA) was carried out on data converted to log values. With differences considered significant at the 95% confidence level: all mucin secretion and cyclic AMP values were significantly different from basal ($P<0.002$); CPT alone was significantly different from Iso alone for mucin secretion and cyclic AMP (*$P<0.008$); IBMX, alone or in combination with Iso, was significantly different from Iso alone for cyclic AMP (**$P<0.002$). The P values denoted in parentheses are from t-tests for nine comparisons of interest (no addition and Iso versus each agonist or combination of agonists). Using Bonferroni correction, a value of 0.0056 (0.05÷9) was considered significant.

AMP response of non-swollen cells or cells swollen in the presence of either non-immune IgG or CFTR antibody are not different (Lloyd Mills *et al*., 1992). At maximally effective concentrations isoproterenol (10 μM) and IBMX (1 mM) alone stimulated mucin secretion to the same extent and this was not increased when both agonists were added together. CPT (1 mM) alone was apparently less effective in stimulating mucin secretion and also did not increase secretion in the presence of isoproterenol. IBMX and CPT increased cyclic AMP levels, but not to the same extent as isoproterenol. However, whereas IBMX potentiated the cyclic AMP rise in response to isoproterenol by 3–4 fold, CPT was ineffective (Table 3). The results indicate that an excessive cyclic AMP rise is not required for correction of CFTR function. Furthermore, an excessive cyclic AMP rise alone is not sufficient to correct the CFTR defect. Thus, DMPX (1 mM), which was ineffective in correcting CFTR function (Table 2) induced a greater cyclic AMP rise alone than did isoproterenol (129.2±7.1% of isoproterenol stimulation; $P<0.01$, $n=4$) and gave a further increase (157.4±12.4%, $P<0.01$, $n=4$) in the presence of isoproterenol. The results show that correction of CFTR function did not correlate with degree of stimulation of mucin secretion, nor with excessive increase in cyclic AMP.

Discussion

A CFTR antibody containing cell has been used for monitoring correction of defective CFTR regulated mucin secretion. Rat submandibular acini provide a good model for studying regulated mucin secretion by the pulse-chase labelling method described. Thus, at least 90% of the radioactively-labelled TCA/PTA insoluble secreted material comigrated with purified bovine submandibular mucin on cellulose acetate electrophoresis and not more than 10% of the radioactivity was lost following digestion with trypsin, chondroitinase ABC or nitrous acid. Although this may only demonstrate that most of the products of digestion have not moved and keratan sulphate chains would be unaffected, nevertheless, the data together with characterization of secreted product on CsCl density gradient centrifugation (Figure 3), strongly indicate that most of the radioactively-labelled secreted material is mucin.

The results show that CPT, an A_1 receptor antagonist, partially corrected defective β-adrenergic stimulation of mucin secretion in CFTR antibody containing cells. However, the mechanism of action is not likely to be adenosine receptor antagonism since a high concentration (1 mM) was required and a similar concentration of DMPX did not correct CFTR function (Table 2). Correction of the CFTR defect also did not correlate with ability to stimulate mucin secretion. Thus, DMPX a potent mucin secretagogue was ineffective, whereas CPT a less potent secretagogue corrected the CFTR mucin secretion defect. IBMX corrected defective CFTR function at a concentration which did not significantly stimulate basal mucin secretion (McPherson *et al*., 1986; Lloyd Mills *et al*., 1992).

Since defective CFTR-mediated mucin secretion was corrected by IBMX and cpt-cyclic AMP, it was postulated that excessive increase in cyclic AMP is the mechanism by which CFTR activity is restored (Lloyd Mills *et al*., 1992). This hypothesis was tested by investigating the effects of the adenosine A_1 and A_2 receptor antagonists on cyclic AMP levels in the presence or absence of isoproterenol. It is clear from the results that CFTR function could be corrected by CPT without potentiation of isoproterenol-induced cyclic AMP rise, although CPT alone increased cyclic AMP levels. Conversely, an excessive increase in cyclic AMP, induced by DMPX in the presence of isoproterenol, was not sufficient to correct defective CFTR function.

The present results have thus demonstrated a lack of correlation between correction of the CF mucin secretory defect and excessive increase in cyclic AMP. Similarly, the effect of xanthines on wild type CFTR Cl^- transport did not correlate with a change in cyclic AMP (Chappe *et al*., 1998). It is not known whether IBMX, which potentiates isoproterenol induced cyclic AMP rise (Bradbury & McPherson, 1988; Quissell *et al*., 1981; Lloyd Mills *et al*., 1992, Table 3) and CPT act by different mechanisms. IBMX activates ΔF508-CFTR Cl^- transport (Drumm *et al*., 1991; Haws *et al*., 1996) and prevents inactivation of CFTR Cl^- channel activity; an effect mimicked by selective protein phosphatase inhibitors (Becq *et al*., 1994). However, lower concentrations of CPT were ineffective in increasing Cl^- transport in CF PAC cells (Guay-Broder *et al*., 1995) and did not activate wild type CFTR Cl^- transport (Chappe *et al*., 1998). IBMX can apparently correct the function of ΔF508-CFTR in severely affected CF submandibular glands (McPherson *et al*., 1986; McPherson *et al*., 1988) in which preliminary evidence suggests a defect in CFTR phosphorylation (Pereira *et al*., 1995). It is possible that an inherent structural feature of the closely related methylxanthines, IBMX and CPT (Figure 6), both of which have an additional larger group substitution and the methyl group at position 1, enables them to interact with

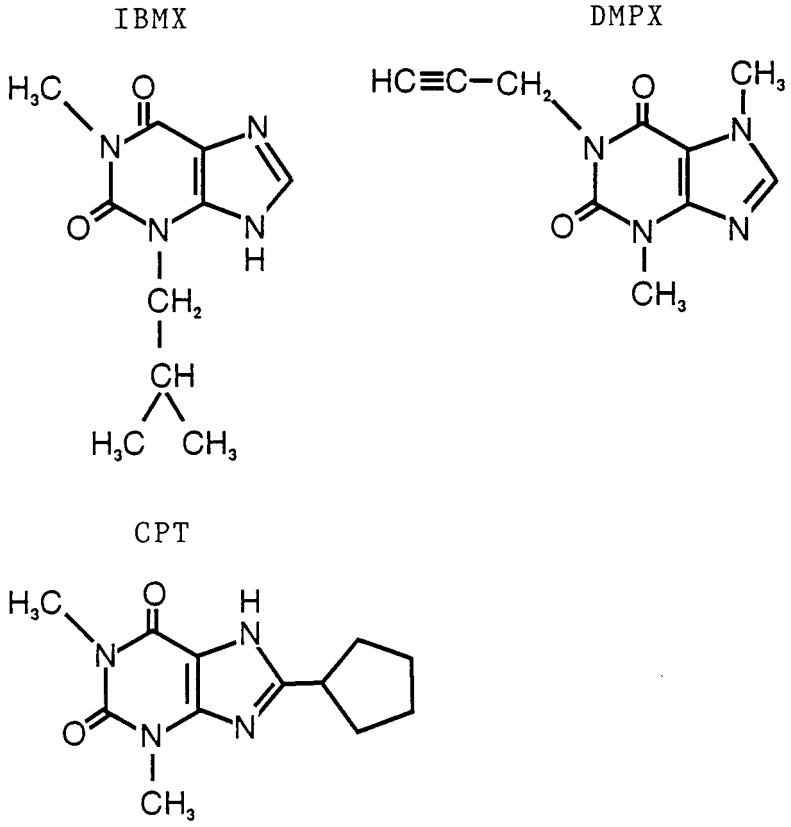

Figure 6 Structure of IBMX, DMPX, CPT.

CFTR itself, thus in the cell model neutralizing the action of the CFTR antibody. In the case of ΔF508-CFTR, there is evidence that at least some of the protein reaches the apical membrane in CF airway epithelial cells (Sarkadi *et al*., 1992; Pereira *et al*., 1995; Morris *et al*., 1998). Whether this is able to be directly activated by IBMX and CPT to give significant effects on CFTR function requires investigation. Another possible mechanism is that a CFTR bypass exists and can be stimulated by IBMX and related compounds. This however seems to be unlikely at least in submandibular glands since cyclic AMP and Ca^{2+}-dependent stimulators of mucin secretion converge *via* a common pathway (Lloyd Mills *et al*., 1991).

It is crucial to determine the mechanism of action of IBMX, CPT and structurally related compounds in correcting CFTR mediated mucin secretion. Restoration of the mucin secreting activity of CFTR is likely to be necessary to alleviate the clinical manifestations of the disease (Dormer & McPherson, 1994; McPherson & Dormer, 1994). The finding, for the first time, that correction can be achieved in the absence of an excessive rise in cyclic AMP levels affords real promise for the development of a rational selective drug treatment for cystic fibrosis which will correct the basic gene protein defect.

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