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A cyclic nucleotide PDE5 inhibitor corrects defective mucin secretion in submandibular cells containing antibody directed against the cystic fibrosis transmembrane conductance regulator protein

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Abstract A selective cyclic nucleotide PDE5 inhibitor corrected the defective mucin secretion response to the β-agonist isoproterenol in submandibular acinar cells inhibited by antibody directed against the cystic fibrosis transmembrane conductance regulator. The PDE5 inhibitor was as effective as cpt-cyclic AMP or a selective PDE4 inhibitor. However, the PDE5 inhibitor had no effect on basal or isoproterenol-stimulated cyclic AMP levels and did not stimulate mucin secretion. The results showing, for the first time, correction of the CFTR mucin secretion defect by a PDE5 inhibitor, which may involve cyclic GMP, will have a major impact in development of a rational drug treatment for cystic fibrosis.

Key words: Cystic fibrosis transmembrane conductance regulator protein; Submandibular; Mucin secretion; Cyclic nucleotide PDE inhibitor

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease characterised by disturbances in ion transport and viscous epithelial mucous secretions [1,2]. The cystic fibrosis gene protein, the cystic fibrosis transmembrane conductance regulator protein (CFTR), acts as a Cl^- channel [3] and we have shown it to be a key regulator of protein secretion [4–8]. Thus, an antibody raised against a synthetic peptide from the first nucleotide-binding domain (NBD) of CFTR inhibited β-adrenergic-stimulated mucin secretion, when introduced into living rat submandibular cells by hypotonic swelling [4]. The cells containing CFTR antibody showed the same phenotype as submandibular gland cells from CF individuals [5–7] and CF mice [8]. In addition, we have shown that the defective β-adrenergic stimulation of mucin secretion in CF cells and in CFTR antibody-containing cells was corrected to the same degree by 3-isobutyl-1-methylxanthine (IBMX), a non-selective cyclic nucleotide PDE inhibitor [4–8]. The CFTR antibody submandibular cell thus provides a good model for investigating correction of the CFTR defect [4,9]. Other studies in agreement showed that an antibody raised against an adjacent peptide sequence in CFTR inhibited cyclic AMP-dependent Cl^- transport [10] and that epithelial cells transfected with CFTR showed increased cyclic AMP-dependent endocytosis and exocytosis [11] and mucin secretion [12,13].

Cyclic nucleotide phosphodiesterases can be classified into seven distinct families, on the basis of their substrate, regulation, and sensitivity to pharmacological agents [14]. The non-selective PDE inhibitor, IBMX, and other more selective PDE inhibitors have been used to investigate whether they activate CFTR-mediated Cl^- transport. IBMX activated Cl^- transport in *Xenopus* oocytes expressing the most common mutant form of CFTR, ΔF508-CFTR, which has a 3-bp deletion leading to loss of a phenylalanine residue [15,16]. An inhibitor of PDE3 (cyclic GMP-inhibited), milrinone, activated Cl^- transport in homozygous ΔF508 CF cells in vitro [17] although IBMX and a PDE4 (cyclic AMP-specific) inhibitor were ineffective [17,18]. Milrinone increased nasal potential difference, which reflects Cl^- transport in vivo, in CF mice [19] but not in CF patients [20]. It is essential to clarify the role of selective PDE inhibitors in restoration of mutant CFTR activity using a well-characterised cell model. Furthermore, evidence suggests that improved CFTR-mediated Cl^- transport, measured by changes in nasal potential difference in vivo, does not necessarily correlate with improvement of CF symptoms [21]. The CFTR-mediated mucin secretion defect is likely to be fundamental to the development of the clinical manifestations of CF and may thus provide a means of evaluating efficacy of new pharmacological agents for CF [22,23]. We have now studied, for the first time, the actions of selective PDE inhibitors on CFTR antibody-inhibited mucin secretion, to investigate the mechanism of correction of defective CFTR function and the involvement of cyclic AMP and cyclic GMP.

2. Materials and methods

2.1. Production of anti-peptide CFTR antibodies

Antibodies were raised against a peptide consisting of 14 amino acids (524–537), residing in the NBD1 region of CFTR [16] and affinity-purified as previously described [4,9]. The peptide sequence used

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator protein; cpt-cyclic AMP, 8-(4-chlorophenylthio)-cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine; KHB, Krebs–Henseleit bicarbonate; KLH, keyhole limpet haemocyanin; NBD, nucleotide-binding domain; PDE, cyclic nucleotide phosphodiesterase

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(CQLEEDISKFAEKD) was unique to human and bovine CFTR (SwissProt database, BLASTP and FASTA3_T, 1997). The rat CFTR sequence (CQLQEDITKFAEQD) has 11 identical and three conserved amino acids; the mouse sequence is identical to rat, but with an additional change (Q at position 5) from rat and human. The antibody has been shown to cross-react specifically with rat and mouse CFTR in native submandibular and pancreatic tissues [4] and with mouse CFTR expressed in CHO cells [9].

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

Procedures were carried out as previously described [4,9,24]. Briefly, acini were pulse-chase labelled with [^{3}H]glucosamine (5 μCi/ml) and suspended in TES-buffered saline (10 mM TES, pH 7.4 containing 143 mM NaCl, 4.7 mM KCl, 1.1 mM $MgCl_2$, 1 mg/ml bovine serum albumin). 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 (approx. 1 mg IgG/ml) was added for 1.5 min at room temperature, followed by washing and resuspension in KHB buffer (see [4,24]) containing 20 mg/ml bovine serum albumin. 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 (Sigma) were dissolved directly into KHB medium at the concentrations used; the selective PDE3, 4 and 5 inhibitors were dissolved initially in DMSO and diluted to give a final concentration of $<1\%$ DMSO in the incubations. An equivalent amount of DMSO was added to control incubations. The PDE5 inhibitor was the least soluble, hence a concentration of 0.2 mM, rather than 1 mM was used. [^{3}H]Glucosamine-labelled mucins, released into the medium at zero time and after 30 min and in aliquots of cells at zero time, 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 [4,9,24]. The radioactively labelled secreted proteins measured in this way have been characterised as mucins [9].

The protein content of cell pellets was determined using the Bio-Rad protein assay kit and mucin release over 30 min expressed as dpm/mg protein or as % basal secretion to take account of variation in unstimulated mucin release between experiments. Isoproterenol secretory responses are relatively consistent within a series of experiments but can vary (see Tables 1, 2 and Fig. 2) between series, as has been published previously [9,25]. Degree of correction was calculated by expressing the difference between isoproterenol response in CFTR antibody-inhibited cells in the presence or absence of PDE inhibitor or cpt-cyclic AMP as a percentage of the difference in isoproterenol response between non-immune IgG and CFTR antibody-containing cells.

Table 1
Actions of PDE inhibitors on mucin secretion and cyclic AMP levels in rat submandibular acini

Addition	Mucin secretion (% basal)	Cyclic AMP (pmol/mg protein)
No addition	100 ± 9	9.4 ± 2.7
Isoproterenol	619 ± 101**	160.3 ± 23.1‼
IBMX	578 ± 84**	27.9 ± 4.9!
PDE3 inhibitor	139 ± 42	11.8 ± 1.7
PDE4 inhibitor	191 ± 20*	168.2 ± 36.6!
PDE5 inhibitor	122 ± 35	4.9 ± 06

Mucin release was measured at 30 min and cyclic AMP at 5 min as described in Section 2, in the presence or absence of isoproterenol (10 μM) or PDE inhibitors (IBMX, PDE3 and PDE4 at 1 mM; PDE5 at 0.2 mM). Mucin release is expressed as % basal, due to the variation in basal secretion between experiments. The range of basal mucin secretion was 414–653 dpm/mg protein. Basal secretion was 3.7 ± 0.3% (mean ± S.E.M., $n=3$) of the total labelled mucin pool, as determined by expressing the amount of labelled mucins in the medium as a percentage of the total labelled mucins in the cells. Results are mean ± S.E.M. for at least three experiments. Significance of differences was assessed by Student's t-test: $^*P<0.002$; $^{**}P<0.001$ for difference from basal mucin secretion; $^!P<0.05$; $^‼P<0.01$ for difference from basal cyclic AMP.

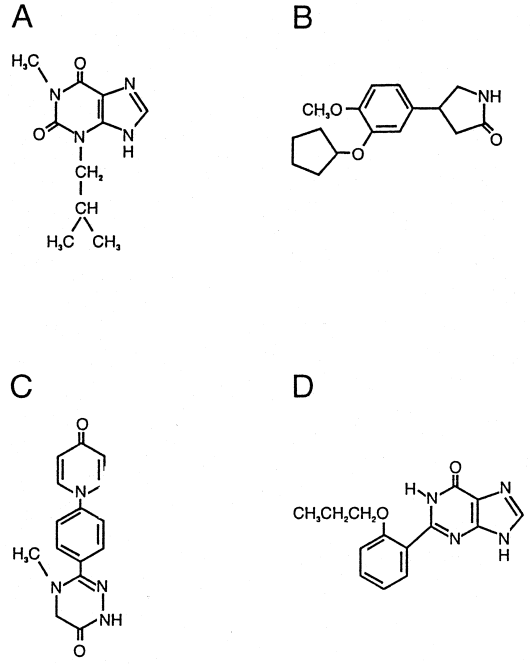

Fig. 1. Structure of PDE inhibitors. A: 3-Isobutyl-1-methylxanthine (IBMX). B: SB 95952 (4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone). C: SB 95654 (*R,S*,-4,5-dihydro-6-[4-(1,4-dihydro-4-oxopyriiny-1-yl)phenyl]-5-methyl-3(2*H*)-pyridazinone). D: SB 96231 (1,7-dihydro-2-(2-propoxyphenyl)-6*H*-purin-6-one).

2.3. Measurement of cyclic AMP and cyclic GMP content

Aliquots of acini suspensions (0.5 ml) were added to an equal volume of ice-cold TCA (20%), extracted and assayed using specific radioimmunoassay kits for cyclic AMP and cyclic GMP (Amersham), as previously described [4].

3. Results

3.1. Structure of selective cyclic nucleotide PDE inhibitors

The mechanism of action of IBMX in correcting defective CFTR function was investigated using selective PDE3 (SB 95654) [26], PDE4 (rolipram, SB 95952) and PDE5 (SB96231) [27] inhibitors. Their structures are shown in Fig. 1 and compared to that of the non-selective PDE inhibitor, IBMX. Of the selective inhibitors used, only SB 96231 possesses a xanthine ring structure but with different side groups, i.e. propoxyphenol at the 2 position rather than the 1-methyl, 3-isobutyl substitutions of IBMX.

3.2. Actions of PDE inhibitors on mucin secretion and cyclic AMP

The actions of selective cyclic nucleotide PDE inhibitors on mucin secretion and cyclic AMP rise from submandibular acini have been compared with maximum responses elicited by the β-adrenergic agonist isoproterenol and the non-selective PDE inhibitor IBMX (Table 1). The PDE3 and PDE5 inhibitors did not significantly increase cyclic AMP or mucin secretion. The PDE4 inhibitor (1 mM) gave the same cyclic AMP rise as isoproterenol but was much less effective in stimulating mucin secretion. By contrast, IBMX (1 mM) stimulated mucin secretion to the same extent as isoproterenol but gave a much lower cyclic AMP rise. The data indicate a lack

Table 2
Actions of PDE inhibitors on isoproterenol-stimulated mucin secretion and cyclic AMP levels in rat submandibular acini

Addition	Mucin secretion (% basal)	Cyclic AMP (pmol/mg protein)
No adddition	100 ± 7	7.9 ± 1.9
Isoproterenol	785 ± 212	373.0 ± 84.0
Isoproterenol+IBMX	924 ± 290	1252.9 ± 129.6*
Isoproterenol+PDE3 inhibitor	692 ± 238	1009.6 ± 91.5*
Isoproterenol+PDE4 inhibitor	708 ± 220	1908.7 ± 150.8**
Isoproterenol+PDE5 inhibitor	755 ± 243	375.5 ± 95.8

Mucin secretion was measured at 30 min and cyclic AMP at 5 min as described in Section 2, in the presence or absence of isoproterenol (10 μM) and PDE inhibitors (IBMX, PDE3 and PDE4 at 1 mM; PDE5 at 0.2 mM), under the conditions shown. Mucin secretion is expressed as % basal, due to the variation in basal secretion between experiments. The range of basal mucin secretion was 232–974 dpm/mg protein. Basal mucin secretion was 3.6 ± 0.3% (mean ± S.E.M., $n = 6$) and isoproterenol-stimulated mucin secretion was 28.3 ± 7.6% (mean ± S.E.M., $n = 6$) of the total labelled mucin pool, as determined by expressing the amount of labelled mucins in the medium as a percentage of the total labelled mucins in the cells. Results are mean ± S.E.M. for six experiments. Significance of differences was assessed by Student's *t*-test. $*P < 0.001$; $**P < 0.0001$ for difference from stimulation by isoproterenol alone.

of correlation between magnitude of increase in cyclic AMP and stimulation of mucin secretion and are in accord with our previous results [25,28].

3.3. Correction of CFTR submandibular mucin secretion response

We have assessed the actions of selective cyclic nucleotide PDE inhibitors in correcting the defective isoproterenol-stimulated mucin secretion in CFTR antibody-containing submandibular cells (Fig. 2) and compared their efficacy to that of cpt-cyclic AMP. PDE4 and PDE5 inhibitors partially corrected the defective mucin secretion response. Although the mean value was lower, the degree of correction by the PDE4 inhibitor was not significantly different from that of the PDE5 inhibitor. The PDE3 inhibitor was ineffective. The PDE5 inhibitor (0.2 mM) was as effective as cpt-cyclic AMP (1 mM) which was previously shown to restore the mucin secretion response of CFTR antibody-inhibited cells to approximately 70–80% of non-immune IgG-containing cells [4]. The current results (Fig. 2) also show that cpt-cyclic AMP restored CFTR antibody-inhibited mucin secretion to 79% (mean) and the PDE5 inhibitor to 75% (mean) of that seen in non-immune IgG-containing cells. Although unswollen cells had a higher mean isoproterenol secretory response than non-immune IgG-containing cells (Fig. 2, legend), the difference was as shown previously [4] not significant.

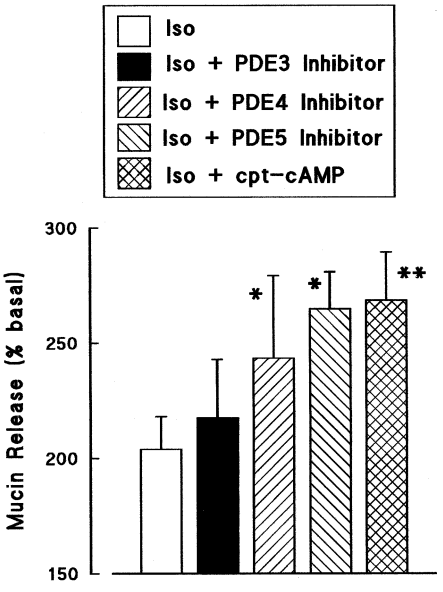

Fig. 2. Correction of CFTR antibody-inhibited β-adrenergic stimulation of mucin secretion by PDE inhibitors. Rat submandibular acini were pulse-chase labelled with [^{3}H]glucosamine and swollen in the presence of CFTR antibody as described in Section 2. Mucin secretion in the presence of isoproterenol (Iso, 10 μM) and PDE inhibitors (PDE3 and PDE4 inhibitors at 1 mM; PDE5 inhibitor at 0.2 mM) or cpt-cyclic AMP (1 mM) as shown, was measured after 30 min. Mucin secretion in response to isoproterenol (10 μM) in unswollen cells was 353.7 ± 45.4 (mean ± S.E.M., $n = 4$) % basal and in cells swollen in the presence of non-immune IgG it was 284.8 ± 42.1 (mean ± S.E.M., $n = 5$; $P < 0.02$ for difference from cells swollen in the presence of CFTR antibody). Results are mean ± S.E.M. for at least four experiments and are expressed as % basal. $*P < 0.05$, $**P < 0.01$ for difference from secretion in the presence of isoproterenol alone as assessed by Student's *t*-test.

3.4. Cyclic AMP rise in the presence of isoproterenol

We have measured correction of the CFTR mucin secretion response in CF cells or CFTR antibody-containing cells in the presence of isoproterenol, since the β-adrenergic response is defective. Thus, effects of PDE inhibitors on cyclic AMP levels in the presence of the β-agonist isoproterenol are shown (Table 2), to indicate whether they potentiate cyclic AMP rise and give increases in cyclic AMP above the levels induced physiologically by β-adrenergic stimulation. Our previous results have shown that the cyclic AMP response of non-swollen cells or cells swollen in the presence of either non-immune IgG or CFTR antibody were not different [4]. None of the selective PDE inhibitors nor IBMX significantly increased mucin secretion above the maximum response induced by isoproterenol alone (Table 2) and the lack of effect was not due to exhaustion of the labelled mucin pool. The results indicate that isoproterenol and the PDE inhibitors stimulate mucin secretion by a common pathway which we have shown to be CFTR-mediated [4,8,9]. However, IBMX, PDE3 and PDE4 inhibitors markedly potentiated the isoproterenol-induced cyclic AMP rise. The PDE3 inhibitor was as effective as IBMX (Table 2), although it did not correct CFTR mucin secretion activity (Fig. 2). By contrast, the PDE5 inhibitor did not potentiate isoproterenol-induced cyclic AMP rise, but corrected defective CFTR function.

3.5. Role of cyclic GMP in correction of defective CFTR function

We have examined the actions of the PDE5 inhibitor on cyclic GMP levels to investigate whether cyclic GMP plays a role in correction of CFTR-mediated mucin secretion. Isopro-

Table 3
Actions of PDE inhibitors on cyclic GMP levels in the presence or absence of isoproterenol

Addition	Cyclic GMP (fmol/mg protein)	
	No isoproterenol	+Isoproterenol (10 μM)
No addition	39.2 ± 1.0 (6)	45.2 ± 1.6 (4)
IBMX (1 mM)	48.1 ± 5.2 (8)	55.6 ± 1.6** (8)
PDE3 inhibitor (1 mM)	39.3 ± 2.4 (3)	47.0 ± 1.8 (4)
PDE4 inhibitor (1 mM)	40.1 ± 1.5 (3)	45.2 ± 2.0 (4)
PDE5 inhibitor (0.2 mM)	47.3 ± 2.5* (5)	64.3 ± 3.6** (5)

Cyclic GMP levels were measured as described in Section 2 following 5 min incubation under the conditions shown. PDE inhibitor concentrations used were 1 mM for IBMX, PDE3 and PDE4 and 0.2 mM for PDE5. Results are mean ± S.E.M. for the number of experiments shown in parentheses. Significance of differences was assessed by Student's *t*-test. $*P < 0.02$ for difference from basal cyclic GMP (no isoproterenol); all conditions in the presence of isoproterenol were significantly different from basal cyclic GMP ($P < 0.05$); $**P < 0.005$ for difference from stimulation by isoproterenol alone.

terenol alone caused a small increase in cyclic GMP, which was further increased in the presence of IBMX or the selective PDE5 inhibitor (Table 3). The PDE5 inhibitor alone also gave a significant increase in cyclic GMP (Table 3). IBMX and the PDE5 inhibitor also increased cyclic GMP levels in the presence of the cholinergic agonist carbamylcholine. Thus, cyclic GMP levels were increased from 32.5, 40.3 ($n = 2$) fmol/mg protein in the presence of carbamylcholine (50 μM) alone to 91.9, 101.4 ($n = 2$) fmol/mg protein in the presence of carbamylcholine (50 μM)+IBMX (1 mM) and to 102.4, 117.0 ($n = 2$) fmol/mg protein in the presence of carbamylcholine (50 μM)+PDE5 inhibitor (0.2 mM). The PDE3 and PDE4 inhibitors did not change cyclic GMP levels in the presence or absence of isoproterenol (Table 3).

The data showing lack of correction of CFTR mucin secretion activity by a PDE3 inhibitor indicate that potentiation of isoproterenol-induced cyclic AMP rise alone is not sufficient to correct CFTR function. In addition, a PDE5 inhibitor that did not increase isoproterenol-induced cyclic AMP rise corrected the CFTR mucin secretion defect and, like IBMX, potentiated the β-agonist-induced cyclic GMP rise.

4. Discussion

We have shown that a selective cyclic nucleotide PDE5 inhibitor partially corrected defective β-adrenergic stimulation of mucin secretion in CFTR antibody-inhibited submandibular cells. The PDE5 inhibitor did not increase cyclic AMP levels, nor did it potentiate isoproterenol-induced cyclic AMP rise. To our knowledge this is the first demonstration that the CFTR mucin secretion defect can be corrected by a PDE5 inhibitor, which increases cyclic GMP but not cyclic AMP levels.

A lack of correlation between magnitude of increase in cyclic AMP and stimulation of mucin secretion in submandibular cells was observed in that isoproterenol and IBMX maximally stimulated mucin secretion, but IBMX gave a much lower cyclic AMP response and a PDE4 inhibitor increased cyclic AMP to the same extent as isoproterenol, but gave much less stimulation of mucin secretion. The data agree with our previous results [25,28] showing dissociation between cyclic AMP rise and mucin secretion and indicate that this is not the sole intracellular messenger involved. The finding that isoproterenol increased intracellular Ca^{2+} in acini populations [29] and that intracellular BAPTA inhibited secretion in response to isoproterenol [25] suggested that intracellular Ca^{2+} may play an important role. Stimulation of wild-type mucin secretion was not a prerequisite for correction of CFTR activity, as shown by the lack of stimulation of mucin secretion by the active PDE5 inhibitor.

Our studies are in agreement with others showing correction of CFTR Cl^- transport by IBMX but not by a PDE3 inhibitor [15,20]. However, other investigators have reported restoration of Cl^- transport by PDE3 but not by IBMX or PDE4 inhibition [17,18]. The present cell model has directly examined correction of isoproterenol-induced CFTR antibody-inhibited mucin secretion. A structurally related compound, 8-cyclopentyl theophylline, also corrected CFTR antibody-inhibited mucin secretion [9] but did not activate wild-type CFTR Cl^- channels [30]. IBMX and the PDE4 inhibitor increased cyclic AMP and potentiated isoproterenol-induced cyclic AMP rise. However, the PDE3 inhibitor showed the same isoproterenol-induced cyclic AMP rise as IBMX but no correction of mucin secretion suggesting that increases in cyclic AMP alone are not sufficient to correct CFTR antibody-inhibited mucin secretion. A similar lack of correlation between increases in cyclic AMP and stimulation of Cl^- transport, induced by PDE3 and PDE4 inhibitors, was observed in a homozygous ΔF508 CF airways epithelial cell line [17].

The finding that a PDE5 inhibitor corrected CFTR antibody-inhibited mucin secretion raised the question as to whether cyclic GMP was involved. The results showing that the PDE5 inhibitor stimulated basal, isoproterenol- and carbamylcholine-induced cyclic GMP levels indicate the presence of an active PDE5 in submandibular acini, although this has yet to be confirmed by direct identification of the enzyme. The role of cyclic GMP in stimulation of protein secretion and ion transport in submandibular acini is not clear. Thus, the present data showed a small elevation of cyclic GMP in response to isoproterenol, which was further increased in the presence of IBMX or a PDE5 inhibitor. However, in agreement with early studies [31,32], the increases were less than those induced by carbamylcholine in the presence of a PDE inhibitor (see Section 3). Although cyclic GMP increases in response to the most potent stimulator of mucin secretion, isoproterenol, are small, it is possible that either cyclic AMP or cyclic GMP can activate CFTR to induce stimulation of mucin secretion. Thus, in addition to the well-established activation of CFTR by cyclic AMP-activated protein kinase [3], the type II, membrane-associated cyclic GMP-activated protein kinase has been shown to phosphorylate CFTR and activate Cl^- transport in rat intestinal cells expressing CFTR [33]. In addition, although the C-type natriuretic peptide, which increased cyclic GMP levels in airways epithelial cells [34], did not stimulate ion transport in human cultured surface epithelial cells [35] it did increase Cl^- transport in mouse nasal epithelium [36] and in a human airways gland cell line [37]. Thus, although correction by the PDE5 inhibitor could be mediated by cyclic GMP, the lack of effect of the PDE4 inhibitor on cyclic GMP (Table 3) suggests that other actions, such as a Ca^{2+}-dependent mechanism or interaction of the PDE inhibitors with CFTR may be involved. The results of the present study suggest that the actions of the selective

PDE5 inhibitor should be investigated on CF mouse and human cells to further elucidate the mechanisms involved and to evaluate their potential for use in CF patients.

The finding, for the first time, that correction of the CFTR mucin secretion defect can be achieved by a selective PDE5 inhibitor is likely to have major impact for CF therapy. Thus, in view of its efficacy as a weak bronchodilator [27], its specificity, in that it does not increase cyclic AMP and it being one of a class of PDE5 inhibitors which includes Viagra®, this would provide a relatively safe compound in the development of a selective pharmacological therapy for cystic fibrosis targeted at the gene protein defect.

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