Effect of IBMX and alkaline phosphatase inhibitors on Cl\(^{-}\) secretion in G551D cystic fibrosis mutant mice

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Cystic fibrosis (CF) pathology is a consequence of mutations in the CF transmembrane conductance regulator (CFTR) protein that functions as an apical membrane Cl\(^{-}\) channel (11). CFTR-mediated Cl\(^{-}\) secretion is markedly reduced in CF, any residual function being dependent on which of the over 400 mutations are considered. Recently, mouse models of CF have become available, including those for two specific human mutations, ΔF508 (6, 28, 30) and G551D (7), the latter representing ~3% of mutant alleles (14). The G551D mutation results in a channel that is localized to the apical membrane but does not allow normal rates of Cl\(^{-}\) secretion in response to ATP binding at the first nucleotide-binding fold (29). Increasing CFTR regulatory domain phosphorylation makes available the second nucleotide-binding fold domain for further ATP binding and increases channel open probability (17). Thus therapeutic strategies could be devised that might activate CFTR by increasing the probability of phosphorylation of those molecules reaching the apical membrane.

Two principal strategies are available: reducing phosphatase activity or reducing adenosine 3',5'-cyclic monophosphate (cAMP) phosphodiester bond hydrolysis (12, 19). Some of the phosphatases associated with CFTR function have been suggested to include protein phosphatase (PP)-2A (5), PP-2C (16), and alkaline phosphatase (4, 25). No evidence for the involvement of PP-2B has been shown to date (15). With respect to CFTR mutant protein, patch-clamp studies have demonstrated that the alkaline phosphatase inhibitor bromotetramisole could induce Cl\(^{-}\) secretion through the ΔF508 protein in precooled simian virus 40-transformed human airway epithelial NP34 cells (4). The mutant CFTR processing is temperature sensitive (8); cooling results in correctly localized protein. However, no Cl\(^{-}\) secretion was seen in ΔF508 airway cells tested with PP-1 and PP-2A inhibitors in combination with the general phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (13). In this latter study, the cells were not precooled, thus resulting in mislocalization of the channel protein. Phosphatase inhibition is likely to be more successful in the case of mutated CFTR that does reach the apical membrane. In keeping with this, a recent patch-clamp study has described the induction of Cl\(^{-}\) secretion in G551D cells using bromotetramisole (4). An alternative strategy for increasing phosphorylation and hence Cl\(^{-}\) secretion is to reduce cAMP phosphodiester bond hydrolysis. Thus IBMX used at high concentrations has been shown to induce Cl\(^{-}\) secretion in Xenopus oocytes transfected with the ΔF508 cRNA (10) (ΔF508 protein is known to reach the apical membrane in Xenopus oocytes). However, in a further study, no secretion could be produced either in cell lines derived from ΔF508 patients or by direct in vivo assessment (13). As for phosphatase inhibition, it is likely that such a strategy may be more successful in the case of a mutated protein reaching the apical membrane in mammalian cells. Thus IBMX (again at
METHODS

Animals

Mice (15-35 g) were housed in a temperature-controlled room (21°C) with food (Special Diet Services, Witham, Essex, UK) and water freely available. MF1 mice were obtained from Harlan. cftr\textsuperscript{G551D} (7) and cftr\textsuperscript{UNC} null (22) mice (producing no CFTR) were generated as previously described and housed on pine shavings. G551D mRNA levels are ∼50% of that of wild-type (+/+ ) levels when assessed in the lung, small intestine, kidney, and testes (7).

In Vivo Measurement

Both these and the in vitro measurements (below) have previously been extensively described (24). Briefly, the reference electrode was placed subcutaneously in a hindlimb of the anesthetized animals [Avertin (tribromoethanol, 0.43 g/kg intraperitoneally)] and connected via N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-Kreb’s buffer of composition (in mM) 140 NaCl, 6 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 glucose, and 10 HEPES (pH 7.4) to a calomel half cell. This was in turn connected to a hand-held computer attached to a preamplifier containing a low-pass signal-averaging filter with a time constant of 0.5 s (Logan Research, Sussex, UK). The exploring electrode consisted of a double-lumen polyethylene tube (outside diameter ∼0.5 mm for nose, 1 mm for rectum), similarly connected to the logging device. Drugs were perfused onto the relevant epithelium at a rate of 40 µl/min. In some cases (in the nasal epithelium), low-Cl\(^{−}\) perfusates were used to increase the driving force for apical Cl\(^{−}\) exit. In this case, NaCl was replaced with equimolar sodium gluconate (final Cl\(^{−}\) concentration 6 mM).

In Vitro Measurements

After in vivo measurements, animals were killed by sectioning of the dorsal aorta, and tracheal, jejunal, and cecal segments were mounted in Ussing chambers of aperture area 0.28 cm\(^2\) (intestinal) and 0.03 cm\(^2\) (airway), under short-circuit conditions. If multiple tissues were obtained from one animal, the mean value was used, so that n refers to the number of animals. Tissues were circulated with Krebs-Henseleit solution of molar composition (in mM) 145 Na\(^{+}\), 126 Cl\(^{−}\), 5.9 K\(^{+}\), 26 HCO\(_3\), 2.5 Ca\(^{2+}\), 1.2 PO\(_4^{3−}\), 1.2 Mg\(^{2+}\), 1.2 SO\(_4^{2−}\), and 5.5 glucose (except for the jejunum where glucose was replaced with equimolar mannitol) using 95% CO\(_2\)-5% CO\(_2\) gas lifts. Short-circuit current (I\(_{sc}\)) was continually recorded, and conductance values were obtained from changes in potential difference produced by a 2-µA pulse.

Fig. 1. Effect of bromotetramisole on in vitro short-circuit current change (ΔI\(_{sc}\)) in wild-type (+/+ ) and G551D tissues. Drugs were administered in presence of amiloride (10 µM). A: tracheal responses in +/+ (n = 6) and G551D (n = 7, except in presence of bromotetramisole n = 2). B: jejunal responses in +/+ (n = 6) and G551D (n = 4). C: cecal responses in +/+ (n = 6) and G551D (n = 3). Shown are effects of bromotetramisole alone (Br; 1 mM), forskolin alone (F; 10 µM), forskolin preceded by bromotetramisole (BrF), ATP preceded by forskolin (FP; 100 µM), and ATP preceded by bromotetramisole and forskolin (BrFP). Error bars indicate SE. *P < 0.05, **P < 0.01, and ***P < 0.001 for comparison of interventions within a genotype. No comparisons or error bars are given for n = 2 data.
nM to 1 mM. No effect was seen on the subsequent forskolin or ATP responses, except at the highest concentration (1 mM), which produced a significant reduction in the response to both agents (Fig. 1A). As in the +/- tissues, no stimulation of current was seen in the G551D tracheae, and again bromotetramisole (1 mM) effectively abolished the subsequent forskolin and ATP responses (Fig. 1A).

Intestinal tract. In +/- tissues, bromotetramisole produced no consistent change in baseline currents at 1 µM–1 mM in either the jejunum (Fig. 1B) or cecum (Fig. 1C). The subsequent response to forskolin was significantly reduced in both tissues at the highest concentration (1 mM) of bromotetramisole. The same profile was seen in the cftrG551D mice, with no induction of Cl\textsuperscript{−} secretion and a complete abolition of the subsequent response to forskolin.

Levamisole

Trachea. Levamisole (100 nM–1 mM) produced no significant change in baseline I\textsubscript{sc} and no effect on the subsequent response to forskolin in +/- mice. Similar results were seen in cftrG551D mice. The subsequent responses to ATP were significantly reduced in G551D tissues (Fig. 2A).

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Intestinal tract. In +/+ mice, levamisole produced no significant effect on the baseline $I_{sc}$ in either the jejunum (Fig. 2B) or cecum (Fig. 2C) at any concentration assessed (100 nM–1 mM). In keeping with this, there was also no significant alteration in the subsequent response to forskolin. Again, in cftr$^{G551D}$ mutant mice levamisole did not induce $\text{Cl}^-$ secretion or enhance the subsequent response to forskolin (Fig. 2, B and C). It is worth noting that the responses in both genotypes to bromotetramisole and levamisole in all tissues were characterized by marked variability, albeit at low absolute levels of response.

**IBMX**

Trachea. In +/+ tissues, IBMX produced a dose-related increase in $I_{sc}$, maximal at 1 mM. The maximal level of stimulation (33.7 µA/cm²) was similar to that seen following addition of forskolin (10 µM; 39.2 µA/cm²) in the absence of IBMX. Furthermore, this stimulation by IBMX (1 mM) was associated with a complete abolition of the subsequent forskolin response (Fig. 3A). Thus, as previously described, it is very likely that the response to IBMX is mediated principally through a cAMP pathway (13). Because very high concentrations of IBMX (5 mM) have been reported to induce $\text{Cl}^-$ secretion in CF tissues, we also assessed the effect of this concentration on +/+ tissues (n = 4). A lower level of stimulation of $I_{sc}$ was seen in comparison with addition of 1 mM, whereas the subsequent response to forskolin was completely abolished and the ATP re-

![Fig. 4. A, B, and C: representative tracheal traces from +/+, cftr$^{G551D}$, and cftr$^{UNC}$ animals, respectively, showing effects of sequential addition of IBMX (IB; bilateral; 1 mM), forskolin (F; mucosal; 10 µM), and ATP (P; mucosal; 100 µM).](http://ajpcell.physiology.org/)

IBMX on forskolin (A; IBF; 10 µM forskolin; mucosal) and, subsequently, ATP (B; IBFP; 100 µM ATP; mucosal) in +/+ (n = 6), G551D (n = 6), and UNC (n = 4) tracheal tissues in vitro. Normal responses to forskolin without IBMX (F; +/+; n = 47; G551D, n = 7; and UNC, n = 5) and to ATP (in presence of forskolin; FP; +/+; n = 34; G551D, n = 7; and UNC, n = 5) are also shown in A and B, respectively. Error bars indicate SE. **P < 0.01, ***P < 0.001, and ****P < 0.0001 for comparison of interventions within a genotype. C: tracheal +/+ in vitro responses to A-23187 (10 µM; mucosal; n = 4) in presence of amiloride (10 µM; mucosal) and forskolin (10 µM; mucosal) and preceded by 1 mM IBMX (IB A-23187; n = 3).
Fig. 6. Effect of IBMX on jejunal $I_{sc}$ in vitro. Drugs were administered in presence of amiloride (10 µM). A: effects on +/+ (n = 8) and G551D (n = 7) jejunal tissues of IBMX alone (IB; 1 mM), forskolin alone (F; 10 µM), and forskolin preceded by IBMX (IBF). Error bars indicate SE. **P < 0.01 and ***P < 0.001 for comparison of interventions between genotypes (IB) and within a genotype (F and IBF). B: representative jejunal traces from +/+ (top trace) and cftrG551D (bottom trace) animals, showing effects of sequential addition of IBMX (IB; 1 mM) and forskolin (F; 10 µM). Effects of glucose (GLU; 10 mM) and phloridzin (PH; 200 µM) to, respectively, stimulate and inhibit Na+-glucose cotransport are also shown.

response significantly diminished. Because of these effects, further studies were carried out at a concentration of 1 mM.

cftrG551D tracheae produced responses to IBMX similar in magnitude to responses of +/+ tissues (Fig. 3B). This was also the case for cftrUNC trachea (n = 3). However, the responses in the mutant mice were transient (Fig. 3B). Typical responses for +/+ and cftrG551D mice are shown in Fig. 4. The subsequent response to forskolin was completely abolished in both mutants (Fig. 5A). It is worth noting that in both these mutant mice forskolin alone produces a response of ~15 µA/cm², which relates to non-CFTR-mediated Cl− secretion (23, 24). In both the cftrG551D and cftrUNC mice, the response to ATP following IBMX was reduced by ~30–50%, although this did not reach significance (Fig. 5B). In contrast, the response to the Ca²⁺ ionophore A-23187 (n = 4) was not altered when added after IBMX (1 mM; n = 3; Fig. 5C).

Jejunum. The +/+ tissue showed a dose-related (1 µM–1 mM) increase in baseline current, with a maximal response very similar to that produced by forskolin (10 µM) in the absence of IBMX (Fig. 6A). A reciprocal reduction in the subsequent forskolin response was seen over the same concentration range. The cftrG551D response to IBMX (1 mM) was 1.8 ± 0.9 µA/cm² (n = 7), and the response to forskolin alone was 0.8 ± 0.9 µA/cm² (n = 7; Fig. 6A). For comparison, the response to IBMX in the cftrUNC mice was 0.6 ± 0.2 µA/cm² (n = 3; not significant compared with cftrG551D), and the response to forskolin alone was 0.5 ± 0.3 µA/cm² (n = 6; not significant compared with cftrG551D). In both types of CF mice, forskolin produced no stimulation of the $I_{sc}$ when added after IBMX. Representative tracings are shown in Fig. 6B.

Cecum. In +/+ tissues, IBMX produced a dose-related increase in $I_{sc}$, again with maximal response similar to that produced by forskolin alone in this tissue (Fig. 7A). This was accompanied by a reciprocal reduction in the response to the subsequent addition of forskolin (Fig. 7A). The response to IBMX in cftrG551D animals was 3.7 ± 0.8 µA/cm² (n = 7), and the response to forskolin alone was 1.9 ± 0.5 µA/cm². For comparison, the response to IBMX in the cftrUNC mice was 0.6 ± 0.4 µA/cm² (n = 3; P < 0.05 compared with cftrG551D), and the response to forskolin alone was −0.2 ± 0.3 µA/cm² (n = 6; P < 0.01 compared with cftrG551D). In both types of CF mice, the subsequent response to forskolin was abolished. Representative tracings are shown in Fig. 7B.

Because of the responses to IBMX, albeit small, seen in the cftrG551D animals, we also assessed the effect of this agent in vivo in the nose and rectum.

Rectum. After amiloride pretreatment, in cftrG551D mice, IBMX produced a response of 1.9 ± 0.9 mV (n = 5) and forskolin alone produced a reduction of −2.1 ± 0.3 mV (n = 7). The forskolin produced no stimulation of the potential difference when added after IBMX. Representative tracings are shown in Fig. 8, A and B.

Nasal cavity. After amiloride pretreatment, IBMX and forskolin increased the post-low-Cl− baseline potential difference of cftrG551D animals (2.5 ± 1.4 mV, n = 4).
This effect was similar in magnitude to that produced by forskolin (10 µM) alone (2.9 ± 1.1 mV, n = 6). A high dose (5 mM) of IBMX (10) was used for these nasal experiments in the light of previous reports of activation of Cl⁻ transport through G551D channels by these doses (5 mM) of IBMX. Representative tracings are shown in Fig. 8, C and D.

**DISCUSSION**

This study demonstrates that in cftr<sup>G551D</sup> mice two agents with alkaline phosphatase-inhibitory activity do not induce Cl⁻ secretion in either the respiratory or intestinal tracts. The phosphodiesterase inhibitor IBMX is able to produce a very small response, likely mediated via CFTR, throughout the intestinal tract. No induction of CFTR-mediated Cl⁻ secretion by IBMX could be measured in the respiratory tract. However, IBMX appeared to activate an alternate, possibly Ca<sup>2+</sup>-related, Cl⁻ secretory pathway in the trachea.

As discussed above, evidence exists to suggest the involvement of a number of phosphatases in the regulation of CFTR. To our knowledge, this has never been assessed in intact tissues or in vivo. We were unable to demonstrate induction of Cl⁻ secretion by bromotetramisole, either in the respiratory or intestinal tract of +/+ animals, assessed at a wide range of concentrations. In addition, no evidence was seen for activation of Cl⁻ secretion in G551D tissues at a concentration (1 mM) previously shown to activate Cl⁻ currents through the G551D protein (4).

Evidence for an effect of bromotetramisole was seen in +/+ intestinal tissues, where addition of the active isomer significantly inhibited the response to the subsequent addition of forskolin; this was not seen following addition of the inactive isomer (data not shown). We also assessed the effect of levamisole, an alkaline phosphatase inhibitor related to bromotetramisole, already in clinical use [for the treatment of roundworm infection (1)], and therefore a potential therapeutic agent for CF. Again no stimulation of basal Cl⁻ secretion was seen in any tissue. Furthermore, this agent did not alter the subsequent response to forskolin. Whether this lack of effect relates to a lower potency for phosphatase inhibition cannot be determined by this study. However, at least from our data in the cftr<sup>G551D</sup> mouse model, neither of the phosphatase inhibitors studied appears to hold promise as a therapeutic agent in CF. We cannot determine from this study whether these alkaline phosphatase inhibitors reached the intracellular surface, but studies have demonstrated their efficacy in cultured epithelial monolayers (18).

We also tested IBMX, an agent having both phosphatase- and phosphodiesterase-inhibitory properties. Initial dosing experiments in +/+ mice suggested that nonspecific effects become apparent at 5 mM, and we therefore used 1 mM in subsequent experiments. Our study suggests that in the respiratory and intestinal tracts of +/+ mice this agent acts principally through a pathway in common with that activated by forskolin. Thus IBMX produced responses of a magnitude similar to those produced by forskolin and abolished the subsequent response to the latter in all tissues studied. These findings, and the marked differences in response to the phosphatase inhibitors, suggest that phosphodiesterase inhibition may be the predominant mode of action of IBMX in these tissues. This is in keeping with many studies demonstrating elevation of intracellular cAMP levels following addition of IBMX, in airway (13) as well as in a variety of other tissues (2, 20).
IBMX has been shown to induce Cl⁻ secretion through ΔF508 mutant CFTR in two patch-clamp studies, but not when assessed in monolayers or in humans in vivo (13). IBMX could also induce Cl⁻ currents through the G551D mutant protein, again when assessed by the patch-clamp technique (4). In the present study, IBMX induced a very low level of Cl⁻ secretion in all three regions of the G551D intestinal tract, whether studied in vitro (jejunum and cecum) or in vivo (rectum). Because we wished to assess the effect of IBMX both on the baseline Iₜₑ, as well as on the subsequent response to forskolin, we could not assess the effect of bumetanide. However, we have previously demonstrated that the forskolin-induced stimulation of Iₜₑ in the murine intestine (both +/- and cftrG551D) is mediated principally through Cl⁻ (7, 24).

Furthermore, in all of the present studies, tissues were pretreated with amiloride. Thus it is likely that the stimulation seen was related to Cl⁻. To assess whether this response was solely mediated through CFTR, we also studied the effect of IBMX in the intestinal tract of null mice. This response provides a background against which to assess the effect of IBMX in G551D tissues. In comparison with the null mice, we consistently saw a small response in the G551D intestinal tract, although this only reached significance in the cecum. The magnitude of this current represents only approximately one-tenth of that induced in the nasal epithelium. This difference may reflect the dominance of CFTR in the apical membrane. Thus under these conditions any response may have remained undetectable.

Interestingly, in the trachea, we observed a response to IBMX that did not differ in magnitude from that seen in +/- mice. However, several features suggest that this response was not principally mediated through CFTR. In +/- animals, forskolin produces an increase in Cl⁻ secretion that relates principally to cAMP-mediated pathways (13, 24). However, approximately one-third of this response is likely to relate to pathways mediated through other second messenger systems, in particular, elevation of intracellular Ca²⁺ (23). We speculate that the response to IBMX was predominantly mediated through this Ca²⁺ pathway in the G551D trachea. In support of this view is our finding that both cftrG551D and null mice exhibited a different IBMX response profile that was significantly more transient than that seen in the +/- mice. In both groups of mutant mice, there is an increased response to Ca²⁺-mediated agonists such as ATP. IBMX reduced the subsequent ATP response in these mutant mice more than in +/- animals, although this did not reach significance. Grubb et al. (13) have suggested this relates to P2 receptor antagonism by IBMX. In keeping with this, there was no effect of IBMX on the subsequent response to the Ca²⁺ ionophore A-23187 (Fig. 5).

Are there therapeutic implications for G551D CF subjects from this study? Intestinal disease in CF subjects is associated in neonates with an ~10% incidence of meconium ileus (27), this being some threefold reduced in the G551D population (14). In addition, intestinal ion transport is altered in CF adults (21), and they are prone to large intestinal obstruction (meconium ileus equivalent). It is unlikely that IBMX would find a role in the treatment of these conditions, even if endoscopic application was feasible at any early stage. Agents such as ATP or UTP that activate alternate Ca²⁺-linked pathways have been suggested as a potential new form of treatment for CF subjects. At least in the cftrG551D mouse model, the magnitude of Cl⁻ secretion produced by IBMX appears to be three- to fourfold less than that produced by ATP. However, ATP was able to produce a response, if somewhat reduced, following IBMX. Thus whether the actions of nucleotides and IBMX are synergistic may deserve further attention. Finally, other phosphodiesterase inhibitors such as theophylline and milrinone are in routine clinical use. Preliminary data indicate that these produce very similar responses to IBMX in +/- tissues, and these and others currently being identified (3) may therefore be worth assessing in CF tissues.

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