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Effects of a new cystic fibrosis transmembrane conductance regulator inhibitor on Cl^- conductance in human sweat ducts

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Effective and specific inhibition of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel in epithelia has long been needed to better understand the role of anion movements in fluid and electrolyte transport. Until now, available inhibitors have required high concentrations, usually in the millimolar or high micromolar range, to effect even an incomplete block of channel conductance. These inhibitors, including 5-nitro-2(3-phenylpropyl-amino)benzoate (NPPB), bumetamide, glibenclamide and DIDS, are also relatively non-specific. Recently a new anion channel inhibitor, a thiazolidinone derivative, termed $CFTR_{Inh}$-172 has been synthesized and introduced with apparently improved inhibitory properties as shown by effects on anion conductance expressed in cell lines and on secretion *in vivo*. Here, we assay the effect of this inhibitor on a purely salt absorbing native epithelial tissue, the freshly isolated microperfused human sweat duct, known for its inherently high expression of CFTR. We found that the inhibitor at a maximum dose limited by its aqueous solubility of 5 μM partially blocked CFTR when applied to either surface of the membrane; however, it may be somewhat more effective from the cytosolic side (~70% inhibition). It may also partially inhibit Na^+ conductance. The inhibition was relatively slow, with a half time for maximum effect of about 3 min, and showed very slow reversibility. Results also suggest that CFTR Cl^- conductance (G_{Cl}) was blocked in both apical and basal membranes. The inhibitor appears to exert some effect on Na^+ transport as well.

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Appreciation of the role of different Cl^--selective anion channels in fluid and electrolyte transport physiology has increased continuously over the past two decades. However, understanding their roles has been handicapped by the lack of an effective, specific inhibitor with activities in the sub or low micromolar concentration range. This void stands in stark contrast to studies of cation channels where potent selective blockers such as amiloride, TEA, glibenclamide, verapamil, nifedipine, and numerous others, have contributed enormously to the understanding of cationic channel functions in physiology and disease. Moreover, understanding anion channels has become even more important with the knowledge that anion channel malfunctions now appear to be the cause of several diseases, including cystic fibrosis (Quinton, 1999), Bartter's syndrome (Simon *et al.* 1997), Dent's disease (Lloyd *et al.* 1996), autosomal dominant and intermediate forms of osteopetrosis (Frattini *et al.* 2003), congenital myotonia (Koty *et al.* 1996), Duchene's muscular dystrophy and autosomal dominant polycystic kidney disease (Persu *et al.* 2000; Devuyst & Guggino, 2002; Jentsch *et al.* 2002).

Unfortunately, the few commonly used inhibitors of anion channels, diphenylamine-2-carboxylate (DPC), NPPB, glibenclamide, stilbenes and bumetanide (Reddy & Quinton, 1999, 2002) are all active only at relative high concentrations and show much overlap in specificity. Recently, the search for potentiators and inhibitors of CFTR, the channel affected in cystic fibrosis, using high-throughput screening led to the discovery of a

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putative CFTR inhibitor, which was found to effectively inhibit CFTR conductance in the submicromolar range in cultured cells (Ma *et al.* 2002). This inhibitor, 3-[(3-trifluoromethyl) phenyl]-5-[(4-carboxyphenyl) methylene]-2-thioxo-4-thiazolidinone, termed $CFTR_{Inh}$-172, blocked the CFTR Cl^- conductance expressed in Fischer rat thyroid (FRT) cells with an IC_{50} of about 300 nM, but at 5 mM it did not affect a Ca^{2+}-activated Cl^- channel in human bronchial epithelial cells or a volume-activated Cl^- channel in FRT cells (Ma *et al.* 2002). As CFTR functions in both secretory and absorptive capacities, we sought to determine the effect of $CFTR_{Inh}$-172 on the function of CFTR natively expressed in a purely absorptive human epithelium, the microperfused human sweat duct.

Methods

Tissue source

Sweat ducts were isolated by micro dissection from full thickness skin biopsies 3 mm in diameter taken from young male volunteer subjects who had given written informed consent. Harvested skin tissue was used immediately or stored in Ringer solution at 4 °C until used experimentally (usually within a few hours, but always within 36 h).

Dissection procedures

Segments of sweat ducts of 1–3 mm in length were identified and isolated from single intact glands harvested by microdissection from the subdermis of the skin.

Microperfusion

Duct segments were then cannulated with double-barrelled microperfusion pipettes so that constant current, 0.5 s pulses, of 50 nA could be passed through one barrel while the transepithelial voltage was measured simultaneously through the other. Luminal perfusing solutions were changed as needed through the voltage-measuring pipette (Quinton, 1986).

Solutions

The luminal and bath Ringer solutions contained (mM): NaCl 140, KCl 5, NaH_2PO_4 3, $MgSO_4$ 1.2, and $CaCl_2$ 1.0. All perfusion solutions were adjusted to pH 7.4 with 1.0 M NaOH. Na^+ conductance was eliminated in the apical membrane, by adding 10 μM amiloride to luminal solutions or replacing Na^+ by K^+ as needed. To impose defined Cl^- gradients, Cl^- was replaced by equimolar concentrations of gluconate in the perifusion or perfusion solutions as needed.

In order to expose the cytosolic surface of the apical membrane directly to the inhibitor, the basilateral membrane (BLM) of the duct was permeabilized by exposure to approximately 2000 units ml^{-1} of α-toxin from *Staphylococcus aureus* (CN Biosciences Inc., CA, USA) for 15–20 min (Reddy & Quinton, 1996). The Ca^{2+} in all Ringer solutions used to perfuse the cytosol was buffered to 80 nM free Ca^{2+} with 2.0 mM EGTA. The pH of these solutions was adjusted to pH 6.8 with 1.0 M KOH, and NaCl and sodium gluconate were replaced by equimolar concentrations of their K^+ salts. After permeabilization, adding 0.1 mM cAMP plus 5 mM ATP to the cytosolic bath reactivated CFTR. To test the effects of $CFTR_{Inh}$-172 on phosphorylation of CFTR, CFTR was stably phosphorylated by adding cAMP in the presence of ATP-γ-S (5 mM) and okadaic acid (1 μM).

$CFTR_{Inh}$-172 was carried in a stock solution of DMSO at 25 mM and added to a final aqueous concentration of 5 μM in the perfusion solutions as needed. Aqueous solutions become saturated at 5 μM (A. Verkman, personal communication). Since complete inhibition of G_{Cl} was never observed, even at the saturated concentration of 5 μM, this concentration was used in all protocols. The inhibitor was a generous gift from Dr Alan Verkman, UC San Francisco.

Statistics

The degree of statistical significance was determined from the Student's t test for paired means of values for pre- and post application of the inhibitor and unpaired means as appropriate. Probability values of less than 0.05 for means were considered to be significantly different. All potentials and conductances are reported as mV and mS cm^{-2}, respectively.

Results

Intact ducts

In ducts microperfused and perifused with symmetrical, isotonic Ringer solution, there was a significant ($P < 0.05$) hyperpolarization of the transepithelial potential (V_t) of nearly −30 mV (from −15 to −43 mV) when $CFTR_{Inh}$-172 was applied in the bath to the contra luminal membrane. Simultaneously, the transepithelial conductance (G_t) fell by almost 50 mS cm^{-2} (from 82 to 34 mS cm^{-2} or 58%). However, it was somewhat surprising that there was only

Table 1. The effect of $CFTR_{Inh}$-172 on the transepithelial potential (V_t) and conductance (G_t) when applied to the apical surface in the lumen and to the basilateral surface in the bath

	NaCl (Lumen)/NaCl (Bath)			
	Lumen $CFTR_{Inh}$-172 ($n = 6$)		Bath $CFTR_{Inh}$-172 ($n = 6$)	
	V_t	G_t	V_t	G_t
Pre	−14.6	82.0	−15.5	82.3
Post	−11.6	53.5	−43.1	34.4
Δ	3.0	28.5	−27.6	47.9
P	0.318	0.008	0.003	0.008

The differences (Δ) between pre- and post application values are shown along with the statistical probability of their significance. At a level of $P \leq 0.01$, only V_t was not different. NaGlu, sodium gluconate.

a slight effect on V_t when $CFTR_{Inh}$-172 was applied in the perfusate to the luminal membrane. The mean V_t depolarized, but not significantly ($P > 0.3$). Nonetheless, the corresponding mean G_t decreased significantly by nearly 30 mS cm^{-2} (from 82 to 54 mS cm^{-2} or 35%; Table 1; Fig. 1).

As depolarization with apical application was unexpected (simple loss of G_{Cl} should have hyperpolarized V_t; Quinton, 1983), we tested whether the decrease in V_t might be due to $CFTR_{Inh}$-172 inhibition of Na^+ transport. We bilaterally replaced Cl^- by the impermeant anion gluconate to remove any effects of G_{Cl} and applied $CFTR_{Inh}$-172 from the lumen (Fig. 2). The mean V_t decreased from −33 to −25 mV, and G_t fell by 21%. On the other hand, when applied from the contra luminal side (Fig. 2), neither V_t nor G_t was significantly altered (Table 2).

To better visualize the inhibitory effects of $CFTR_{Inh}$-172 on G_{Cl} in the apical membrane and to examine potential effects of membrane polarization on inhibitor efficacy, we imposed steep Cl^- diffusion gradients in both directions across the epithelium by replacing Cl^- by gluconate first in the lumen and then in the bath. After applying $CFTR_{Inh}$-172 to the luminal surface, when the Cl^- gradient was from bath to lumen, the mean V_t depolarized from −75 to −54 mV (28%) and the mean G_t fell by 27% (Table 3; Fig. 3). When the gradient was reversed, the V_t decreased from +31 to −1 mV and G_t fell by 48% (Table 4).

We then applied $CFTR_{Inh}$-172 to the contra luminal side of the duct epithelium in the bath and again tested the effect of membrane polarization on inhibitory efficacy. We found that when the Cl^- gradient was from bath to lumen, the mean V_t depolarized from −58 to −45 mV and G_t fell by 27% (Table 3). When the gradient was reversed, the mean V_t completely reversed polarity from +27 mV to −22 mV and G_t fell by 51% (Table 4; Fig. 3).

Permeabilized ducts

In order to determine whether $CFTR_{Inh}$-172 could permeate the basilateral membrane and inhibit apical membrane CFTR from its cytoplasmic surface, we applied $CFTR_{Inh}$-172 to BLM permeabilized ducts to ensure that the inhibitor had access to the cytosolic surface of the membrane. Normally, after permeabilization with α-toxin, CFTR conductance in the apical membrane becomes nil, but can be restored by phosphorylation in the presence of cAMP and ATP (Reddy & Quinton, 1992). Therefore, after activating CFTR with cAMP (10 μM) and ATP (5 mM), we tested the application of $CFTR_{Inh}$-172 on each side of the membrane in the presence of a bath-to-lumen Cl^- gradient. The possibility of a confounding effect from Na^+ conductance (G_{Na}) was avoided by replacement of Na^+ by K^+ in all solutions and/or by adding amiloride (10 μM) to the luminal perfusate. When $CFTR_{Inh}$-172 was applied in the luminal perfusate, the potential across the apical membrane (V_A) fell from 40 to 29 mV and G_t decreased by 41%. When $CFTR_{Inh}$-172 was added to the cytosolic bath, V_A fell from 54 to 21 mV and the apical conductance (G_A) decreased by 73% (Table 5).

$CFTR_{Inh}$-172 was equally effective in inhibiting CFTR that was stably phosphorylated or 'locked open' (Reddy & Quinton, 1994) by prior stimulation of endogenous protein kinase A (PKA) with cAMP (10 μM) in the presence of ATP-γ-S (5 mM) and the phosphatase inhibitor okadaic acid (1 μM).

Discussion

Properties of the sweat duct model for drug testing

The sweat duct should be an excellent model for testing the effects of drugs on CFTR function since it naturally expresses possibly the highest known density of CFTR channels in its apical membrane (Cohn *et al.* 1991; Kartner *et al.* 1992). It also expresses a high CFTR Cl^- conductance in its basal membrane (Reddy & Quinton, 1989*b*). Its reabsorptive function is crucially dependent on CFTR as demonstrated by the hereditary disease cystic fibrosis, in which the channel is defective. Furthermore, the apical membrane appears to be characterized by only two ion conductances, a Na^+ conductance through the epithelial Na^+ channel (ENaC) and a Cl^- conductance through CFTR (Bijman & Quinton, 1987; Reddy & Quinton, 1989*a*,*b*). Similarly, the basilateral membrane is characterized mainly by a

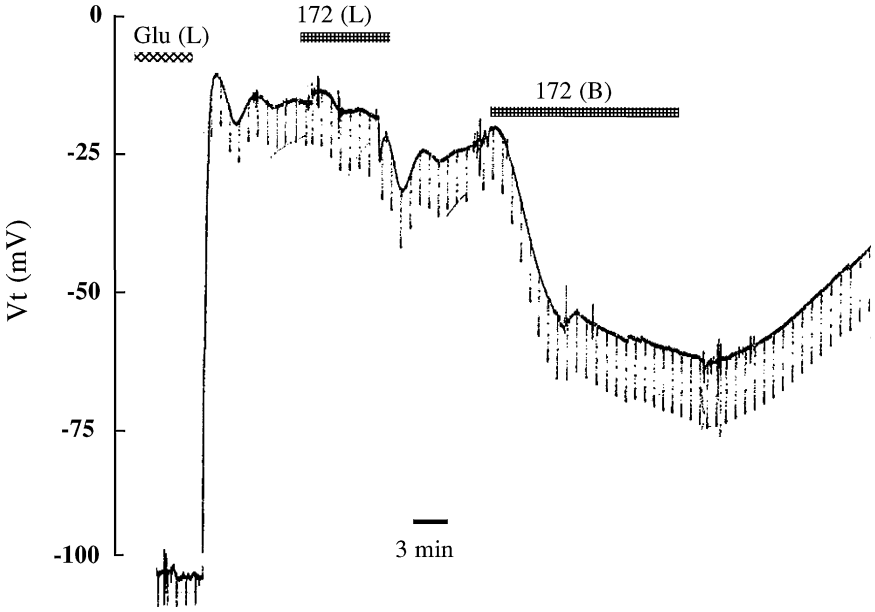

Figure 1. Effect of $CFTR_{Inh}$-172 applied on the luminal and basilateral side of the intact sweat duct
After application in the luminal perfusate, in contrast to expectations for blocking the G_{Cl} independently of G_{Na}, V_t did not hyperpolarize, but G_t fell, suggesting simultaneous inhibition of both G_{Na} and G_{Cl} in the luminal membrane. In contrast, when applied on the serosal side, V_t hyperpolarized markedly suggesting that blocking G_{Cl} in the BLM unmasked its K^+ potential. The lumen (L) and bath (B) of the ducts were perfused continuously with NaCl-containing Ringer solution, except as indicated initially when the lumen contained sodium gluconate (Glu).

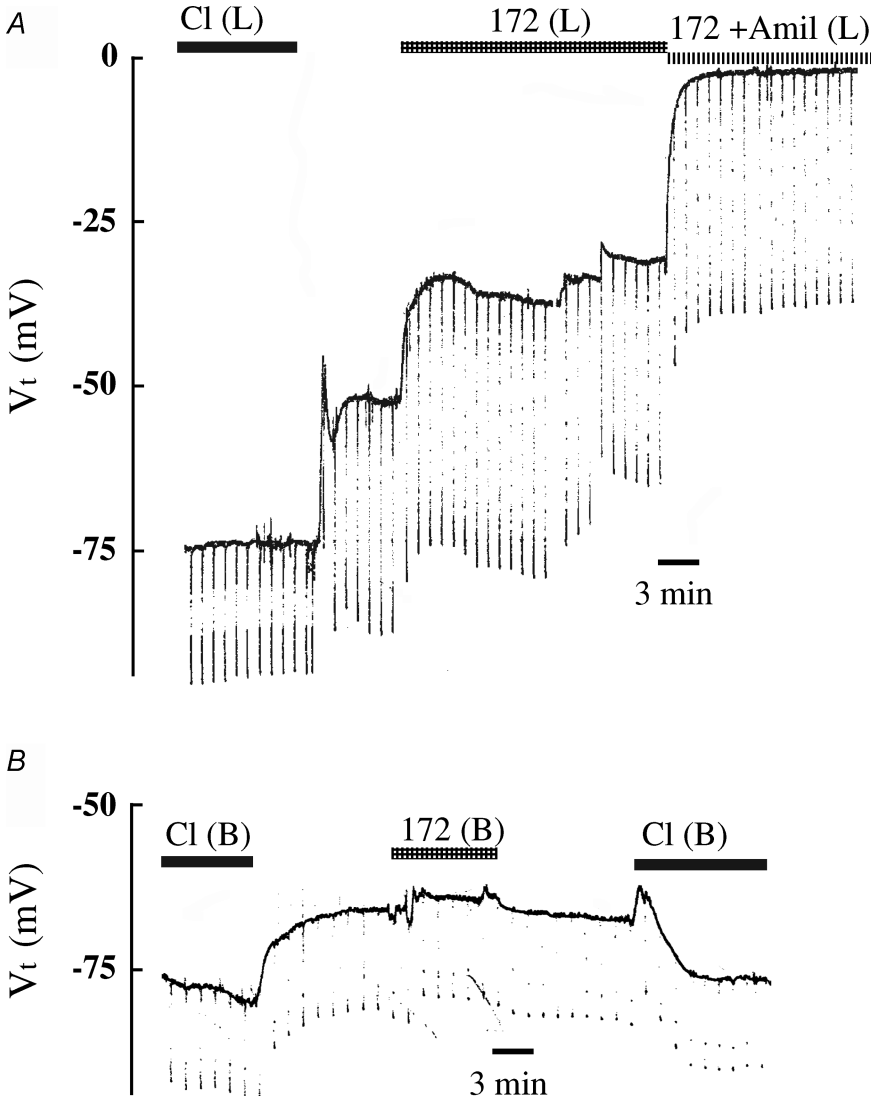

Figure 2. Effect of $CFTR_{Inh}$-172 on luminal and basilateral side of the intact sweat duct in the absence of Cl^-
A, trace shows that completely removing Cl^- from the tissue by replacing luminal Cl^- by gluconate, depolarized V_t from about −73 mV to about −50 mV, which should reflect the potentials due to Na+ transport alone. Adding $CFTR_{Inh}$-172 (5 μM) to the luminal solution further depolarized V_t to about −35 mV suggesting that the inhibitor also blocked G_{Na}. Further addition of amiloride, essentially abolished V_t. *B*, when $CFTR_{Inh}$-172 (5 μM) was added to the bath side alone, virtually no effect was seen on the Na^+ transport potential. The bath (B) and lumen (L) were continuously perfused with sodium gluconate except as indicated: Cl (NaCl), 172 ($CFTR_{Inh}$-172), Amil (amiloride).

Table 2. The effect of $CFTR_{Inh}$-172 on the transepithelial potential (V_t) and conductance (G_t) when applied to the apical surface in the lumen and to the basilateral surface in the absence of Cl^-

	NaGlu (Lumen)/NaGlu (Bath)			
	Lumen $CFTR_{Inh}$-172 ($n = 6$)		Bath $CFTR_{Inh}$-172 ($n = 6$)	
	V_t	G_t	V_t	G_t
Pre	−33.3	26.5	−38.8	21.1
Post	−25.1	21.0	−33.4	17.6
Δ	8.2	−5.5	5.4	−3.5
P	0.003	0.008	0.256	0.202

The differences (Δ) between pre- and post application values are shown along with the statistical probability of their significance. $CFTR_{Inh}$-172 had no significant ($P > 0.05$) effect when applied to the basilateral side on either V_t or G_t. NaGlu, sodium gluconate.

Table 3. The effect of $CFTR_{Inh}$-172 on the transepithelial potential (V_t) and conductance (G_t) when applied to the apical surface or to the basilateral surface in the presence of a Cl^- gradient from bath to lumen

	NaGlu (Lumen)/NaCl (Bath)			
	Lumen $CFTR_{Inh}$-172 ($n = 6$)		Bath $CFTR_{Inh}$-172 ($n = 7$)	
	V_t	G_t	V_t	G_t
Pre	−75.1	23.0	−58.4	33.5
Post	−54.1	16.8	−44.6	24.6
Δ	21.0	−6.2	13.8	−8.9
P	0.010	0.012	0.007	0.009

The differences (Δ) between pre- and post application values are shown along with the statistical probability of their significance. At a level of $P \leq 0.02$, $CFTR_{Inh}$-172 had a significant ($P \leq 0.05$) effect on both V_t and G_t when applied to either surface. NaGlu, sodium gluconate.

K^+ and a Cl^- conductance (Reddy & Quinton, 1991) so that the effects of the inhibitor on CFTR can be studied in virtual isolation from other conductances. In isotonic NaCl Ringer solution, microperfused ducts from normal subjects generally exhibit a spontaneous transepithelial potential difference of about -10 ± 5 mV (lumen negative with respect to contra luminal bath) with a constitutively high Cl^--dependent transepithelial conductance of about 100 mS cm^{-2} (Quinton, 1986). The fact that the sweat duct in patients with cystic fibrosis has virtually no Cl^- conductance provides a unique and excellent model to compare and evaluate the effects of conductance inhibitors on native functioning human CFTR. Ducts from patients with cystic fibrosis that inherently express dysfunctional CFTR also exhibit spontaneous lumen negative potentials of −60 to −110 mV (Quinton & Bijman, 1983) and a G_t of only about 15 mS cm^{-2} (Quinton, 1986). If Cl^- in solutions perfusing normal ducts is replaced by an impermeant anion such as gluconate, the transepithelial potential strongly hyperpolarizes in the direction of the Cl^- diffusion gradient and G_t falls markedly; however, cystic fibrosis ducts are largely unresponsive to these imposed gradients (Quinton, 1986; Quinton & Reddy, 1989). This is to say that efficient, specific pharmacological inhibition of CFTR conductance should 'convert' the normal duct to a cystic fibrosis duct; that is, inhibition of normal anion conductance should mimic the native state of ducts from cystic fibrosis patients.

Limitations

While the preparation demonstrates many advantages, it is limited in its ability to render absolute measures of the specific conductance of the tissue. This limitation derives mainly from the variations among the duct tubules and the strict theoretical dependence of the mathematical solution for tissue conductance on the diameter of the lumen. The specific resistance of the duct epithelium was calculated from the cable equation for determining the electrical resistance of an isolator surrounding a conductive core. In our case, the isolator is the duct tubular epithelium and the core is the conductive luminal perfusate (Greger, 1981). Due to the inability to precisely measure the luminal diameter (12–20 μm) of the intact tubule under transmitted light, precision is not better than $\pm$ 1–2 μm in measuring the luminal diameter, which results in some uncertainty of the measure of G_t. On the other hand, the measure of V_t depends only on the electrochemical forces and permeability properties of the membrane. As such, it is independent of structural variations. Therefore, measured changes in V_t may be a more consistent index of changes in G_{Cl} than measured changes in G_t.

Effects of $CFTR_{1nh}$-172 on salt transport

We were surprised to find that application of $CFTR_{Inh}$-172 to the lumen of the normal sweat duct had no significant, detectable effect (Table 1; Fig. 1). V_t did not hyperpolarize in contrast to what we would have predicted from the loss of apical Cl^- conductance demonstrated in cystic fibrosis ducts. (For example, in ducts from patients with cystic fibrosis, where no or little CFTR is expressed, the spontaneous V_t was about −70 mV in bilateral isotonic NaCl Ringer solution.) On the other hand, after $CFTR_{Inh}$-172, G_t decreased by about 35% (Table 1; Fig. 1). As this result might be compatible with inhibition of

Na^+ transport activity, we examined the effects of the inhibitor on the duct in the absence of Cl^- (replaced by gluconate). With bilateral sodium gluconate, no G_{Cl} or transepithelial electrolyte diffusion gradient exist so that V_t reflects the underlying electromotive forces for Na^+ absorption driven by active transport (essentially the sum of the apical membrane Na^+ EMF and the basilateral K^+ EMF). Under these conditions, when added to the luminal perfusate, both V_t and G_t fell significantly (25% and 21%, respectively; Table 2; Fig. 2) and when added to the bath, neither value was affected. These results suggest that in addition to CFTR, the inhibitor may have an inhibitory effect on active Na^+ transport by blocking ENaC from the luminal side.

We have shown previously that G_{Na} depends upon a functioning, conductive CFTR, so that if G_{Cl} is inhibited, we might expect G_{Na} to fall accordingly. The partial loss of conductances from both Na^+ and Cl^- pathways would have less apparent effect on membrane selectivity and voltage than if G_{Na} remained independent of G_{Cl}. This prediction seems consistent with the present results of decreased overall ductal conductance without a significant change in V_t after luminal application of $CFTR_{Inh}$-172 in bilateral NaCl with no transepithelial ion gradients present. This result and explanation assumes that the inhibitor may be relatively less permeable through the apical membrane.

In contrast, bath application of $CFTR_{Inh}$-172 to the basilateral surface caused striking hyperpolarization of the epithelium in bilateral NaCl Ringer solution. In this case the hyperpolarization of V_t may be explained by shifting the BLM to a predominantly K^+-selective membrane. Blocking G_{Cl} in the BLM should hyperpolarize this membrane by shifting it towards the K^+ equilibrium potential (Reddy & Quinton, 1991). Since V_t is the difference between basal membrane voltage (V_b) and V_A, the loss of BLM G_{Cl} is reflected in a much more negative V_t. Moreover, the results in the permeabilized duct suggest that $CFTR_{Inh}$-172 is more effective from the cytosolic side. Of course, we cannot exclude the (likely) possibility that the inhibitor permeates the BLM of non-permeabilized ducts and not only blocks the G_{Cl} in the BLM, but also blocks the apical membrane CFTR G_{Cl} from the cytosolic surface. Still, while this may be the case, complete inhibition is not realized because the hyperpolarization of V_t due to the inhibitor was low compared to what we expect for a near complete block of both membranes as exemplified by cystic fibrosis ducts lacking CFTR. Cystic fibrosis ducts bathed in bilaterally NaCl spontaneously exhibit V_t of *ca* −75 mV (Quinton, 1983) compared to the mean V_t of −43 mV seen here after adding $CFTR_{Inh}$-172 to the bath.

Effects of polarization on $CFTR_{Inh}$-172 inhibition

We then questioned whether the electrical polarity of the membrane might affect the inhibitor's ability to interact with CFTR. As CFTR G_{Cl} is large and constitutively open in the intact duct, the bath-to-lumen Cl^- diffusion potential markedly hyperpolarized V_t (−10 to −75 mV, lumen negative). Application of $CFTR_{Inh}$-172 to the duct lumen or to the bath depolarized the diffusion potential to about the same degree and decreased G_t by about the same amount (Table 3), suggesting that under these conditions the effect was about equal on either side of the tissue.

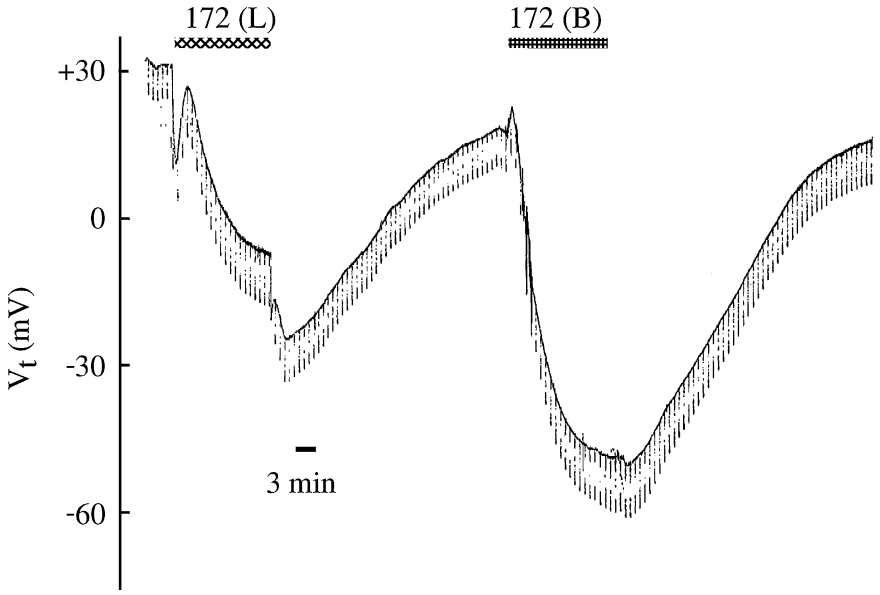

Figure 3. Effect of $CFTR_{Inh}$-172 on the luminal and basilateral side of the intact sweat duct
$CFTR_{Inh}$-172 (5 μM) was applied first in the luminal and then in the bath perfusate. Addition of the inhibitor induced a slow, reversible inhibitory effect on V_t. The duct was continuously perfused with NaCl in the lumen (L) and sodium gluconate in the bath (B). 172, ($CFTR_{Inh}$-172).

Table 4. The effect of $CFTR_{Inh}$-172 on the transepithelial potential (V_t) and conductance (G_t) when applied to the apical surface or to the basilateral surface in the presence of a Cl^- gradient from lumen to bath

	NaCl (Lumen)/NaGlu (Bath)			
	Lumen $CFTR_{Inh}$-172 ($n = 3$)		Bath $CFTR_{Inh}$-172 ($n = 3$)	
	V_t	G_t	V_t	G_t
Pre	30.5	77.9	26.5	68.7
Post	−1.0	47.6	−21.5	33.9
Δ	−31.5	−37.3	−48.0	−34.8
P	0.008	0.006	0.015	0.048

The differences (Δ) between pre- and post application values are shown along with the statistical probability of their significance. At a level of $P \leq 0.05$, $CFTR_{Inh}$-172 had a significant effect on both V_t and G_t when applied to either surface.

Table 5. The effect of $CFTR_{Inh}$-172 on the *trans* apical membrane potential (V_A) and conductance (G_A) of basilaterally permeabilized ducts when applied to the apical surface or to the cytosolic surface in the presence of a Cl^- gradient from lumen to bath

	BLM permeabilized: KCl (lumen)/KGlu (cytoplasmic)			
	Lumen $CFTR_{Inh}$-172 ($n = 6$)		Cytosol $CFTR_{Inh}$-172 ($n = 7$)	
	V_A	G_A	V_A	G_A
Pre	40.2	16.8	53.6	21.9
Post	29.1	10.0	21.8	5.9
Δ	−11.1	−6.9	−31.8	−16.0
P	0.049	0.014	0.001	0.013

The differences (Δ) between pre- and post application values are shown along with the statistical probability of their significance. $CFTR_{Inh}$-172 had a significant effect on both V_t and G_t when applied to either surface. BLM, basilateral membrane; KGlu, potassium gluconate ($P \leq 0.05$).

We then reversed the Cl^- diffusion gradient by replacing Cl^- in the bath by gluconate. When $CFTR_{Inh}$-172 was added to either the lumen or the bath, V_t hyperpolarized and G_t also fell about equally (Table 4). As the inhibitory effect was about equal with application to either the cytosolic or luminal surface under polarized and depolarized conditions ($P > 0.05$), we surmise that there is little, if any, electrostatic effects on drug distribution. That is, electrical gradients, which favoured carrying a higher (or lower) concentration of the inhibitor (negative charged) into the membrane, did not appear to alter its effect.

Even so, the results seem to suggest that the inhibitory effect is larger when Cl^- is in the lumen (Tables 3 and 4). The apparently larger effects with luminal Cl^- may be simply due to higher diffusion potentials and larger conductances that are present with Cl^- in the lumen.

Effect of $CFTR_{Inh}$-172 on the cytosolic membrane surface

To examine the effectiveness of the inhibitor on the cytosolic surface of CFTR, we applied the inhibitor alternately to the apical surface and then to the cytosolic surface of basilaterally permeabilized ducts after activating CFTR with cAMP and ATP. In the presence of a lumen-to-cytosol Cl^- gradient of 150 mM, luminal $CFTR_{Inh}$-172 reduced V_A by 11 mV and G_A by 7 mS cm^{-2}. The inhibitor seemed somewhat more effective when applied to the cytosolic side, reducing V_A by 32 mV and G_A by 16 mS cm^{-2}.

Time course and reversibility

Inhibition of CFTR G_{Cl} with $CFTR_{Inh}$-172 was relatively slow, requiring more than 10 min to read half-maximal effect ($T_{1/2} = 3.1 \pm 0.4$ min) to approach maximal effect. Likewise, even though the inhibition appears to be highly, if not fully reversible, washout required an even longer time course ($T_{1/2} = 17.0 \pm 2.6$ min; Figs 1, 3 and 4 show traces of V_t and V_A revealing the slow inhibitory effect and slow reversibility). These rates of action seem similar to those first reported for transfected FRT cells (Ma *et al.* 2002).

Effect on phosphorylation

$CFTR_{Inh}$-172 might inhibit the phosphorylation activation of the Cl^- channel instead of CFTR itself. However, this appears not to be the case. At least, if $CFTR_{Inh}$-172 inhibits phosphorylation, it must also directly inhibit CFTR as well. If its only action were to inhibit phosphorylation, it is unlikely that $CFTR_{Inh}$-172 would have inhibited CFTR after it had been stably phosphorylated and its endogenous phosphatases blocked with okadaic acid (Quinton & Reddy, 1994); however, it did (Fig. 4).

Relative effectiveness

At its aqueous saturated concentration of 5 μM, $CFTR_{Inh}$-172 appears to block as much as 70% of CFTR G_{Cl} when applied to the cytosolic side of the membrane (Table 5; Fig. 4). Its inhibitory efficacy from the luminal surface may be less, although it seemed equally effective on either side of the intact duct when Cl^- gradients were present. Even

so, it is encouraging that compared to previously examined inhibitors of G_{Cl} in the sweat duct, $CFTR_{Inh}$-172 appears to be a more potent blocker. We note that bumetanide and DIDS required 1 mM (also near saturation points) or nearly 20 times the concentration of $CFTR_{Inh}$-172 used here to block 85% and 70% of CFTR G_{Cl}, respectively. These inhibitors were also most effective from the cytosolic side. Other commonly used anion conductance inhibitors were even less potent (Reddy & Quinton, 1999).

Lastly, we note with some consternation that the inhibitory effects seen here in native tissue are not nearly as complete as they appeared to be in amphotericin B-permeabilized FRT monolayers. The inhibition of Cl^- conductance was reflected by the virtual short circuit current (I_{sc}) that arose after a 2:1 Cl^- gradient (150:75 mM) was imposed across the cells. The current was monitored as a function of inhibitor concentrations up to 5 μM where virtual current was almost completely blocked. It is not clear why CFTR in cultured cells should be more sensitive than in native tissue. On the other hand, in non-permeabilized primary cultures of human bronchial epithelial cells, blocked with amiloride, the putative secretory current appeared to be reduced from about 10 to 3 μA (*ca* 70%; see Figure 3b in Ma *et al.* 2002), which is more in line with our findings here. However it is interesting that in contrast to the sweat duct, $CFTR_{Inh}$-172 seemed to be more effective from the apical than from the basal surface in bronchial cells. This difference may be related to the secretory function of bronchial cells, which presumably exhibit CFTR only in the apical membrane compared to the exclusively absorptive function of the duct, which exhibits CFTR in both membranes.

Conclusion

Overall, $CFTR_{Inh}$-172 blocks CFTR G_{Cl} in the apical and basal membrane of the human reabsorptive sweat duct and probably does so by direct interaction. Unfortunately, the limited solubility of $CFTR_{Inh}$-172 prevents determination of a maximal inhibitory effect. $CFTR_{Inh}$-172 may be useful in identifying Cl^- channels, particularly if it proves to be highly selective for CFTR. However, it suffers from a low aqueous solubility, slow interaction and reversibility rates, and possible effects on Na^+ transport.

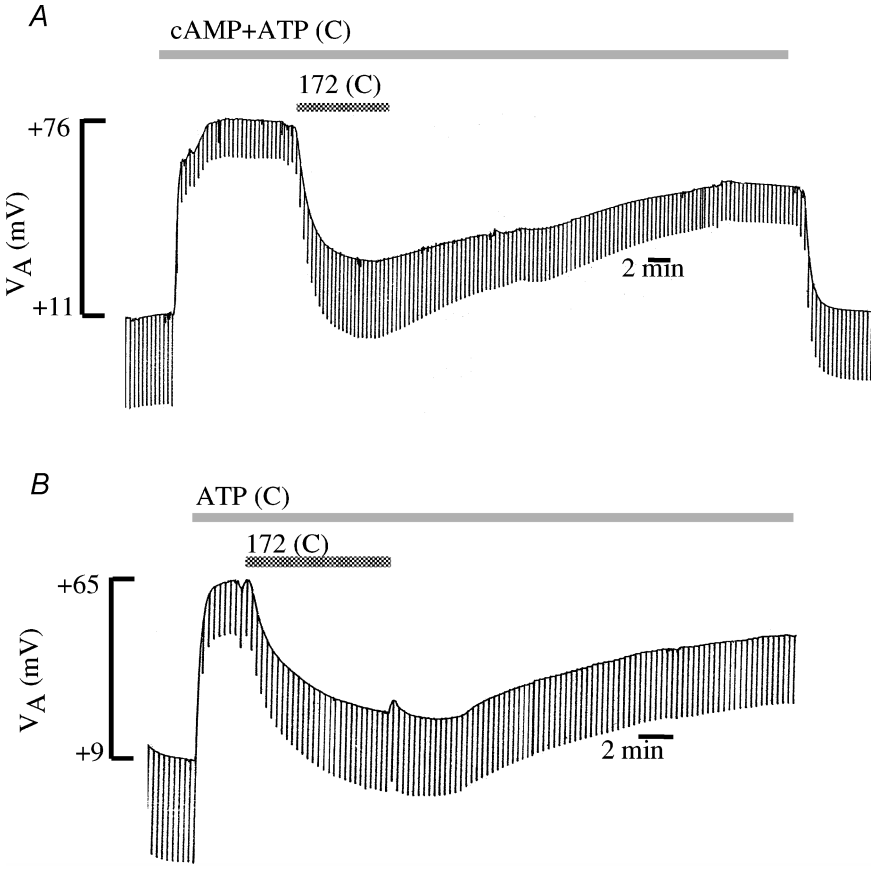

Figure 4. Lack of effect of stably phosphorylating CFTR on $CFTR_{Inh}$-172 inhibition
A, effect of the inhibitor on CFTR activated by cAMP (10 μM) + ATP (5 mM) on the cytoplasmic side of the apical membrane after α-toxin induced permeabilization of the basilateral membrane. *B*, similar experiment, except that CFTR was stably phosphorylated by prior cAMP-dependent phosphorylation in the presence of ATP-γ-S (5 mM). After activating CFTR Cl^- conductance by adding ATP, $CFTR_{Inh}$-172 continued to effectively inhibit CFTR Cl^- conductance, indicating that it may act directly on CFTR. The ducts were continuously perfused with NaCl and amiloride (10 μM) in the lumen and potassium gluconate and the phosphatase inhibitor, okadaic acid (1 μM) in the bath.

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