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Effect of Anion Transport Blockers on CFTR in the Human Sweat Duct

M.M. Reddy, P.M. Quinton
Department of Pediatrics, UCSD School of Medicine, University of California, San Diego, La Jolla, CA-92093-0831, USA

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Abstract. Cystic fibrosis transmembrane conductance regulator (CFTR) is a protein kinase A (PKA) and ATP regulated Cl^- channel. Studies using mostly *ex vivo* systems suggested diphenylamine-2-carboxylate (DPC), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and glybenclamide inhibit CFTR Cl^- conductance (CFTR G_{Cl}). However, the properties of inhibition in a native epithelial membrane have not been well defined. The objective of this study was to determine and compare the inhibitory properties of the aforementioned inhibitors as well as the structurally related anion-exchange blockers (stilbenes) including 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) in the microperfused intact and basilaterally permeabilized native sweat duct epithelium. All of these inhibitors blocked CFTR in a dose-dependent manner from the cytoplasmic side of the basilaterally permeabilized ducts, but none of these inhibitors blocked CFTR G_{Cl} from the luminal surface. We excluded inhibitor interference with a protein kinase phosphorylation activation process by "irreversibly" thiophosphorylating CFTR prior to inhibitor application. We then activated CFTR G_{Cl} by adding 5 mM ATP. At a concentration of 10^{-4} M, NPPB, DPC, glybenclamide, and DIDS were equipotent and blocked ~50% of irreversibly phosphorylated and ATP-activated CFTR G_{Cl} (DIDS = 49 ± 10% > NPPB = 46 ± 10% > DPC = 38 ± 7% > glybenclamide = 34 ± 5%; values are mean ± SE expressed as % inhibition from the control). The degree of inhibition may be limited by inhibitor solubility limits, since DIDS, which is soluble to 1 mM concentration, inhibited 85% of CFTR G_{Cl} at this concentration. All the inhibitors studied primarily blocked CFTR from the cytoplasmic side and all inhibition appeared to be independent of metabolic and phosphorylation processes.

Key words: Sweat duct — CFTR — DPC — NPPB — Glybenclamide — DIDS — SITS — DNDS — PKA — ATP

Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is a PKA- and ATP-regulated Cl^- channel [1, 26, 40]. The CFTR Cl^- channel plays a central role in transepithelial Cl^- absorption and secretion. Physiological significance of these ion channels is demonstrated by the facts that excessive stimulation of these Cl^- channels by bacterial toxins can cause life-threatening diarrhea [39] and functional abnormalities associated with CFTR Cl^- channels cause severe pathology in cystic fibrosis (CF) [25, 36, 39, 40].

Diphenylamine-2-carboxylate (DPC), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and glybenclamide have been widely used as blockers of CFTR Cl^- channels [2, 4, 5, 10, 12, 14, 20, 37]. Mostly, these studies were conducted either on heterologous systems transfected with CFTR [9, 12, 14, 20, 36, 39] or on epithelial cell lines [14, 21]. Little is known about the efficacy and the mechanism of action of these CFTR Cl^- channel blockers on endogenous CFTR expressed in a native epithelium. Most of the effects of some of these blockers on CFTR Cl^- currents were studied while applying the inhibitors to the extracellular surface of intact cells. It was not clear from these studies whether these inhibitors blocked CFTR from the extracellular side or from the cytoplasmic side after diffusion through the cell membrane. The effects of these inhibitors on endogenous CFTR expressed in a native epithelial membrane are not well characterized, either.

Correspondence to: P.M. Quinton; email: pquinton@ucsd.edu

Furthermore, questions remain as to whether the reported effects of these inhibitors on CFTR are direct or are an indirect consequence of the primary effects on the process of CFTR regulation. For example, DPC was reported to inhibit transepithelial Cl^- conductance in tracheal epithelium by diminishing intracellular cAMP levels due to its inhibition of prostaglandin synthesis [41]. NPPB was shown to be a metabolic inhibitor causing reduced ATP levels in phagocytic cells [18, 19]. Glybenclamide was shown to inhibit a number of cellular enzymes including PKA [8, 23].

The stilbene compounds DIDS, SITS and DNDS are structurally related to glybenclamide, which was shown to block CFTR [6, 13] and ATP-sensitive K^+ channels [39]. Since these compounds have been commonly used as blockers of anion exchangers [6] as well as certain Cl^- channels [3, 11, 16, 22], we sought to determine the relative inhibitory effects of these agents on CFTR in a native epithelial tissue. This study characterizes inhibition of CFTR G_{Cl} by arylaminobenzoates (DPC, NPPB), sulfonylurea (glybenclamide), and disulfonic stilbenes (DIDS, SITS, DNDS) in the freshly isolated, microperfused human sweat duct epithelium. Using intact and basilaterally α-toxin-permeabilized ducts we determined the sidedness of the effect of the inhibitors on the process of CFTR regulation. We showed that all the inhibitors studied blocked CFTR only from the cytoplasmic side, and that inhibition appeared to be independent of phosphorylation activation or metabolic effects.

Materials and Methods

Tissue Acquisition

Sweat glands were obtained as previously described [34] from adult male volunteers without medical history who gave informed consent. The isolated glands were transferred to a cuvette with Ringer's solution cooled to 5°C where the segments of reabsorptive duct (~1 mm in length) were separated from the secretory coil of the sweat gland under microscopic control (Nikon model SMZ-10). Using a very small, special glass pipette, the sweat duct was transferred to a perfusion chamber containing Ringer's solution for cannulation and micro perfusion at 35 ± 2°C.

Selective Permeabilization of the Basilateral Membrane

The basilateral membrane of the sweat duct was selectively permeabilized with a pore-forming agent (1,000 units/ml of α-toxin derived from *Staphylococcus aureus*) in cytoplasmic Ringer's solution containing 140 mM KGlu (potassium gluconate) and 5 mM ATP applied to the basilateral surface of the microperfused sweat duct for 15 to 30 minutes. As described earlier [26], α-toxin effectively removes the basilateral membrane as a barrier to ions and small solutes such as cAMP and ATP without affecting the functional integrity of the apical membrane. This preparation allowed free manipulation of intracellular cAMP and ATP (cofactors and substrate for PKA phosphorylation) so that the properties of the regulation of CFTR-G_{Cl} in the apical membranes can be examined apart from functions of the basilateral membrane and from the influence of uncontrolled, small cytosolic solutes.

Electrical Measurements

Electrical Setup

After cannulating the lumen of the sweat duct with a double lumen cannula made from theta glass (1.5 mm diameter, Clark Electromedical Instruments, Reading, UK), a constant current pulse of 50–100 nA for a duration of 0.5 seconds was injected through one barrel of the cannulating pipette containing NaCl Ringer's solution. The other barrel of the cannulating pipette served as an electrode for measuring transepithelial potential (V_t) with respect to the contraluminal bath and as a cannula for perfusing the lumen of the duct with selected solutions. V_t was monitored continuously using one channel of a WPI-700 dual electrometer referenced to the contraluminal bath. Transepithelial conductance (G_t) was measured as described earlier [26, 28, 34] using the cable equation to derive the specific membrane conductance from the amplitude of transepithelial voltage deflections in response to transepithelial constant current pulses (50–100 nA).

Apical Cl^- Conductance (G_{Cl})

Cl^- diffusion potentials (V_{Cl}) and G_{Cl} were monitored as indicative of the level of activation of G_{Cl}. Following α-toxin permeabilization of the basilateral membrane, the epithelium is simplified to a single (apical) membrane with parallel Na^+ and Cl^- conductances [26, 28, 34]. Application of amiloride further simplified the system into a predominantly Cl^--selective membrane. The composition of Ringer's solution in bath and lumen was designed to set up a single ion gradient, i.e., exclusively for Cl^- [140 mM KGlu (bath)/150 mM NaCl (lumen)]. Under these conditions, V_t and G_t can be regarded as closely reflecting V_{Cl} and G_{Cl}, respectively.

Solutions

The luminal perfusion Ringer's solutions adjusted to pH 7.4 contained (in mM) NaCl, 150; K, 5; PO_4, 3.5; $MgSO_4$, 1.2; Ca^{2+}, 1; and amiloride, 0.01. The cytoplasmic bath solution contained K, (145), gluconate (Glu 140); PO_4, 3.5; $MgSO_4$, 1.2; and Ca^{2+}, 0.26, buffered with EGTA 2.0 mM to 80 nM free Ca^{2+}, adjusted to pH 6.8. The impermanent anion gluconate was used to replace Cl^- in Cl^--free Ringer's solution. ATP (5) and cAMP (0.01) were added to the cytoplasmic bath as needed. Phosphatase inhibitors fluoride (5), vanadate (0.001) and okadaic acid (0.001–0.00001) were added to the cytoplasm as a phosphatase inhibition cocktail (PIC). We achieved stable phosphorylation of CFTR by activating it in the presence of 10^{-5} M cAMP, 5 mM ATP-γ-S, and the phosphatase inhibition cocktail [27, 32]. We confirmed stable phosphorylation of CFTR by subsequent activation of CFTR G_{Cl} by adding 5 mM ATP alone without cAMP.

We tested the effects of a range of concentrations of the inhibitors: diphenylamine-2-carboxylate (DPC; 10^{-6} to 10^{-3} M), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; 10^{-6} to 10^{-4} M), glybenclamide (10^{-7} to 10^{-4} M), DIDS (10^{-6} to 10^{-3} M), SITS (10^{-6} to 10^{-3} M), and DNDS (10^{-6} to 10^{-3} M). Solutions containing DPC (stock solution contained 50 mg/ml of DPC in methanol), NPPB (stock solution contained 0.01 M NPPB in ethanol) and Glybenclamide (stock solution contained 0.05 M in ethanol) were prepared from previously mixed stock solutions. DIDS and SITS were

directly stirred into solutions as needed. All inhibitors were mixed either in NaCl Ringer (to test the effect of these agents on the basilateral or on the apical membranes of intact sweat ducts) or in K Gluconate Ringer with or without cAMP and ATP (to test inhibitor effects on the cytoplasmic surface of the apical membrane of basilaterally permeabilized ducts). The highest concentration of each inhibitor used approached its limit of solubility.

Data Analysis

The transepithelial conductance in the presence and absence of cAMP + ATP were standardized to 100% (when CFTR G_{Cl} was activated) and 0% (when CFTR G_{Cl} was deactivated), respectively. The relative effects of inhibitors on V_{Cl} and G_{Cl} were evaluated in terms of the percent inhibition of the level of CFTR G_{Cl} when it was maximally activated by cAMP + ATP or by ATP alone after irreversible phosphorylation of CFTR. The data is presented as the mean of percent inhibition ± SE (where n = total number of ducts from a minimum of 3 human subjects). Statistical significance was determined on the basis of Student's t-test for paired samples. A P value of <0.05 was taken to be significantly different.

Results

We studied the effect(s) of three different classes of compounds on CFTR G_{Cl} in the sweat duct, which included: 1) two arylaminoalkyl benzoates (NPPB and DPC); 2) a sulfonylureal (glybenclamide); and 3) two disulfonic stilbenes (SITS and DIDS). We studied the effect of these inhibitors when applied to 1) the basilateral or apical (luminal) membrane of intact microperfused ducts and 2) the cytoplasmic side of the basilaterally permeabilized ducts before and after irreversibly phosphorylated CFTR [27, 32].

Effects on the Intact Duct

We tested the effects of the anion transport inhibitors on the intact ducts microperfused with 150 mM NaCl BSS (basic salt solution) in the lumen and bath (serosal side). There was no effect of any of the inhibitors from the luminal surface (Figs. 1 & 2), and only DIDS and NPPB only slightly affected the V_t and G_t when applied in the bath (Figs. 2 & 3).

Basilateral application of DIDS (1 mM) induced a small increase in V_t and transepithelial resistance (Fig. 3). However, under identical conditions, NPPB caused a small decrease in V_t along with a small increase in resistance (Fig. 2).

For comparison, we applied amiloride in the lumen (Na gluconate and K gluconate in lumen and bath, respectively), to inhibit the Na^+ channel (ENaC) and abolish transepithelial potential and increase transepithelial resistance of permeabilized ducts (Fig. 4). The fact that the inhibitors did not alter the electrical properties of the duct indicates that they probably had no effect on ENaC either (Fig. 1–4).

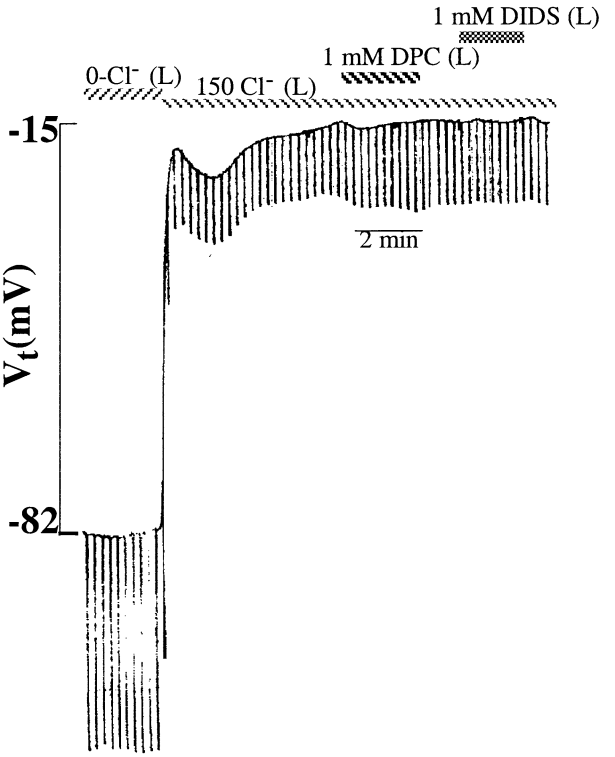


Fig. 1. Lack of effect of DPC and DIDS on the luminal surface of the intact sweat duct. At the beginning of the experiment the tissue integrity is indicated by the large Cl^- diffusion potential. Notice that DPC (10^{-3} M) and DIDS (10^{-3} M) had no significant effect on either the apical Na^+ or Cl^- conductances. Luminal and bath perfusates contained 150 mM NaCl.

Effect on the Permeabilized Ducts

After permeabilizing the basilateral membrane with α-toxin, we blocked Na^+ conductance with 10^{-5} M amiloride in the luminal perfusate, and tested the effect of each inhibitor separately on G_{Cl} before and after stable phosphorylation of CFTR [27, 32].

Before Stable Phosphorylation of CFTR

DPC (Fig. 5), NPPB (Fig. 6), glybenclamide (Figs. 7 and 8), SITS (Fig. 9), and DNDS (Fig. 5), all blocked cAMP- and ATP-activated CFTR G_{Cl} to varying degrees when applied to the cytoplasmic bath after permeabilizing the basilateral membrane with α-toxin. However, except for SITS (which almost completely blocked CFTR G_{Cl} activity, as shown Fig. 9), none of the compounds tested completely blocked CFTR G_{Cl} at maximal concentrations tested.

After Stable Phosphorylation

To determine whether an inhibitor interrupted phosphorylation of CFTR, we applied the inhibitors to stably phosphorylated CFTR in ducts when G_{Cl}

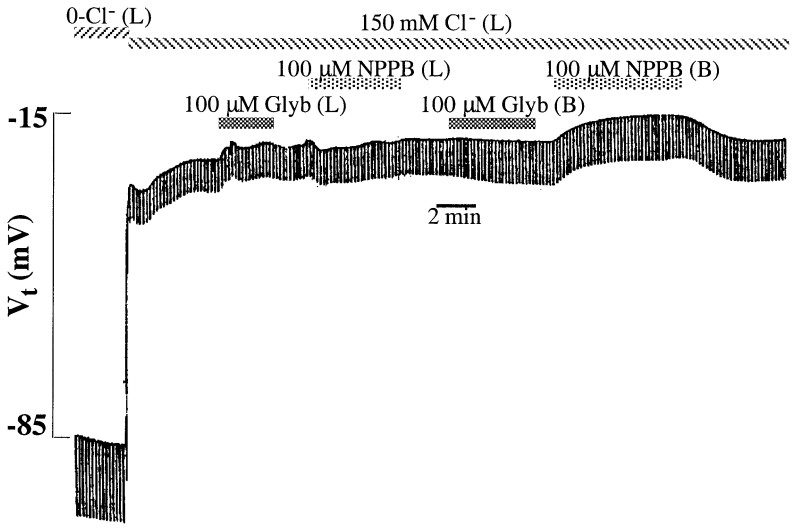


Fig. 2. Effect of glybenclamide and NPPB on the intact sweat duct. NPPB or glybenclamide (100 μM each) was applied either to the bath or to the luminal perfusate. Glybenclamide on either the serosal or luminal side had little effect on the transepithelial electrical properties of the intact duct. Similarly, NPPB had no detectable effect from the luminal side. However, NPPB in the bath induced a small, but discernible depolarization of transepithelial potential accompanied by a small increase in transepithelial resistance as shown by a small increase in the voltage deflections due to transepithelial current pulses (50 nA). The tissue viability was indicated by the large Cl^- diffusion potential induced by luminal Cl^- substitution with impermeant gluconate. The lumen and bath were otherwise perfused with 150 mM NaCl.

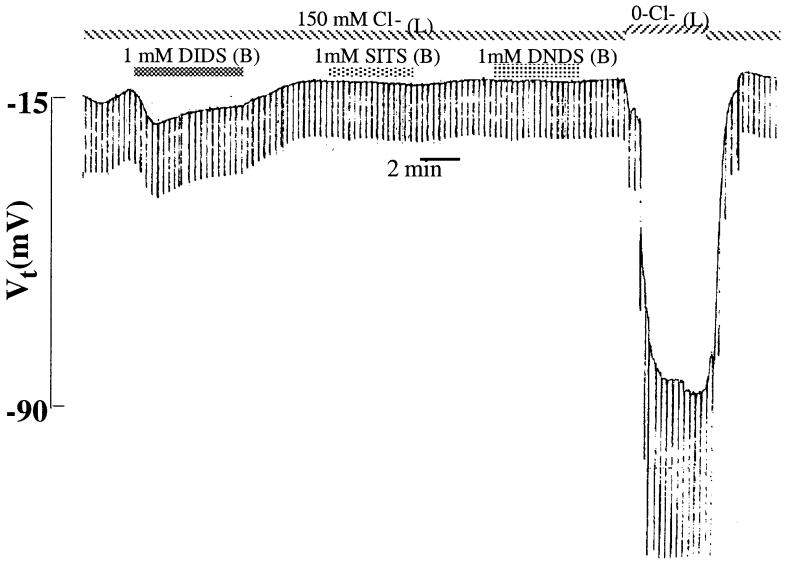


Fig. 3. Effect of stilbenes on the serosal side of the intact duct. In this experiment the serosal bath was perfused with Ringer's solution containing DIDS, SITS or DNDS (1 mM each). Except for a small hyperpolarization of V_t after application of DIDS, these stilbenes had little effect on the transepithelial electrical properties of sweat duct. The tissue integrity is a function of an apical CFTR G_{Cl}, which is indicated by the presence of significant apical CFTR G_{Cl}, as indicated by a large Cl^- diffusion potential generated by substituting luminal Cl^- with impermeant anion gluconate.

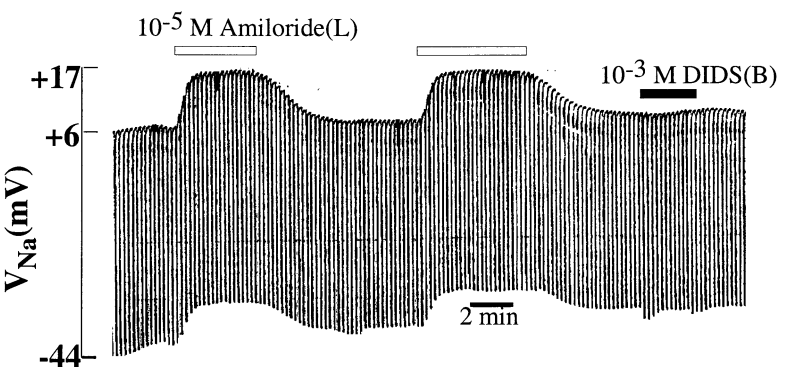


Fig. 4. Effect of amiloride and DIDS in the cytoplasm on apical ENaC in a permeabilized duct. This experiment shows for comparison that inhibiting ENaC with amiloride (10^{-5} M) in the lumen characteristically depolarizes the transepithelial potential and decreases transepithelial conductance (the lumen and cytoplasmic bath contained 150 mM NaGlu and 150 mM KGlu, respectively). The lack of effect of the inhibitors (as in Fig. 2) indicates the lack of effect of these compounds on the lumen to cell Na^+ diffusion potential (V_{Na}) and hence on ENaC. The basilateral membrane of this duct was permeabilized with α-toxin, to ensure that DIDS (10^{-3} M) from the cytoplasmic side had no effect on ENaC.

was activated by ATP alone. We then tested for the inhibitory effect(s) of the aforementioned anion transport blockers. Preliminary studies showed that the degree of acute inhibitory effects of DPC, NPPB, and glybenclamide before stable phosphorylation of CFTR was comparable to the effect of the same inhibitor on CFTR G_{Cl} after stable phosphorylation of CFTR.

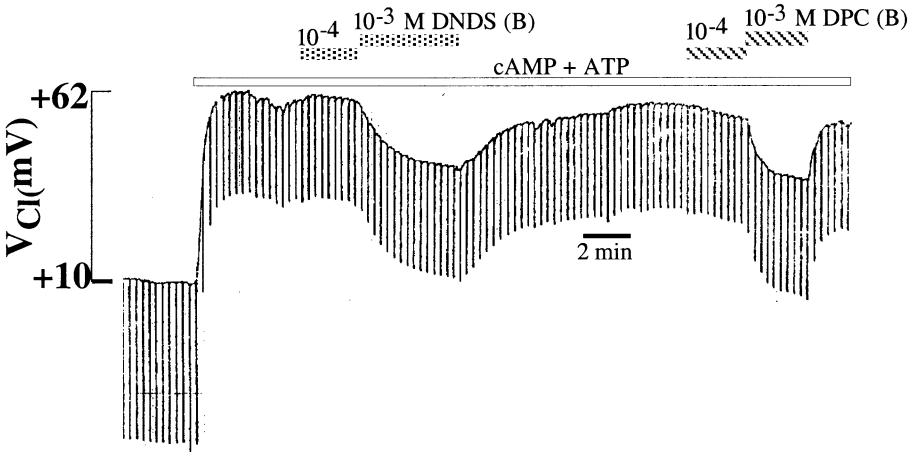


Fig. 5. Effect of cytoplasmic DNDS and DPC on CFTR G_{Cl}. DNDS (10^{-3} M) and DPC (10^{-3} M) caused a small, reversible inhibition of activated CFTR G_{Cl}. DNDS and DPC at $< 10^{-4}$ M concentration had no detectable effect on CFTR G_{Cl} in ducts after permeabilizing the basilateral membrane. Lumen and bath were perfused with NaCl and KGluconate Ringers.

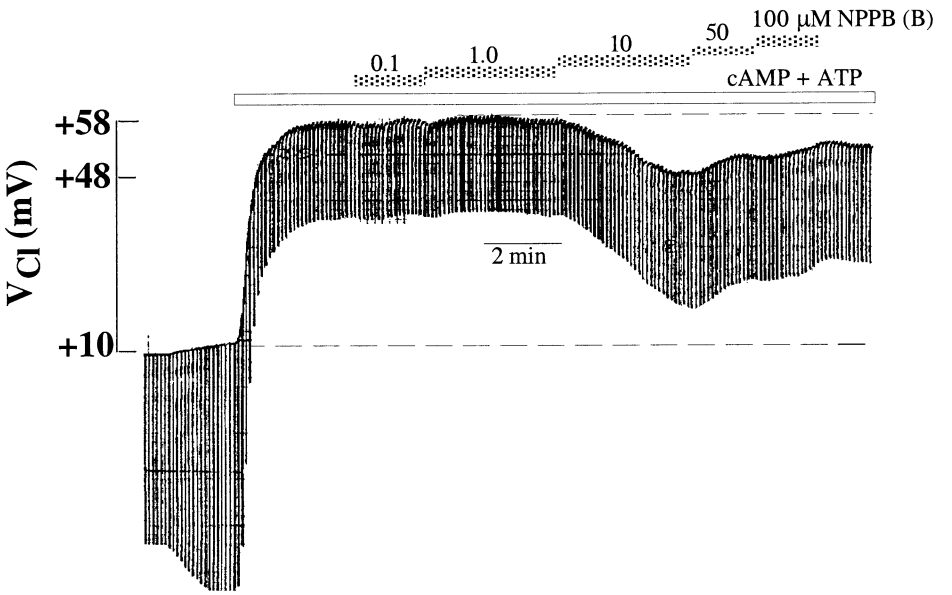


Fig. 6. Effect of cytoplasmic NPPB on activated CFTR G_{Cl}. NPPB (0.1-100 μM) in the cytoplasm had a small, but significant, inhibitory effect on CFTR G_{Cl}. Preparation and perfusion solutions as in Fig. 5.

DPC. We tested the effect of 10^{-6} to 10^{-3} M DPC in the cytoplasmic bath on the G_{Cl} activity of stably phosphorylated CFTR activated with 5 mM ATP. The percentage inhibition of CFTR G_{Cl} at increasing inhibitor concentrations ranged from 14.9 ± 5.2% (10^{-6} M) to 52.2 ± 11.2% (10^{-3} M). The effect of DPC was relatively fast (< 2 min) and fully reversible upon washout (Fig. 10).

NPPB. Application of NPPB to the cytoplasmic bath after stable phosphorylation and ATP activation of CFTR also resulted in a low-affinity inhibition of the G_{Cl} activity. The percentage inhibition of CFTR G_{Cl} at increasing inhibitor concentrations ranged from 8 ± 9% (10^{-6} M) to 46 ± 10% (10^{-4} M). NPPB inhibition of CFTR G_{Cl} was fully reversible following washout of the inhibitor (Fig. 11).

Glybenclamide. Glybenclamide inhibited stably phosphorylated and ATP-activated CFTR G_{Cl} by 11 ± 7% (10^{-7} M) to 34 ± 5% (10^{-4} M). Glybenclamide inhibition was completely reversible following washout (Fig. 12).

DIDS. Among the inhibitors studied, we found that the stilbene DIDS was the most effective inhibitor of CFTR G_{Cl}. In contrast to an almost complete lack of effect from the luminal side, application of DIDS in the cytoplasmic bath produced the greatest

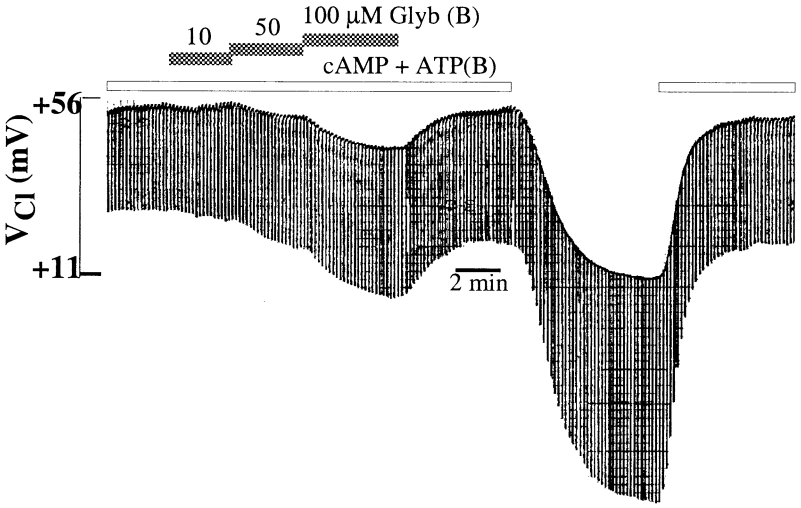


Fig. 7. Dose response of glybenclamide inhibition on activated CFTR G_{Cl}. The effects of increasing concentrations of glybenclamide in the cytoplasm on (cAMP + ATP) activated CFTR in the apical membrane of a permeabilized duct is shown. Prior to the application of glybenclamide, CFTR G_{Cl} activation by cAMP and ATP was indicated by the large lumen-positive Cl^- diffusion potential, which was abolished following the washout of cAMP and ATP. Inhibition of CFTR was detectable at 50 μM. Preparation and perfusion solutions as in Fig. 5.

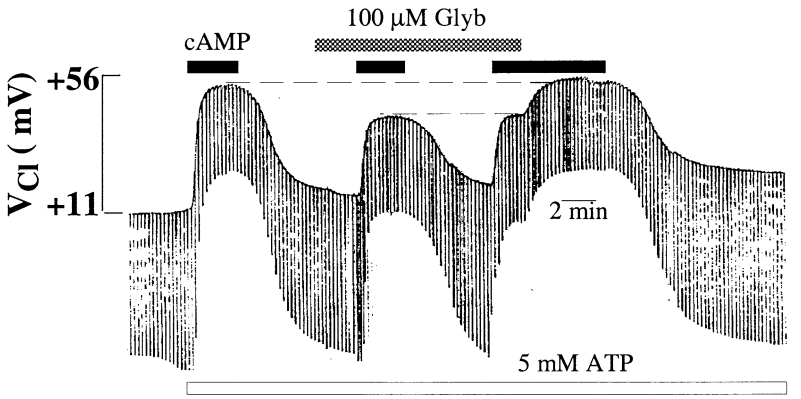


Fig. 8. Effect of the cytoplasmic glybenclamide on CFTR G_{Cl}. Application of glybenclamide before activating CFTR caused a small, but significant, inhibition of CFTR G_{Cl}. Glybenclamide did not affect deactivation of CFTR G_{Cl} following cAMP washout showing no effect of the inhibitor on phosphatase dephosphorylation of CFTR. Notice that the glybenclamide effect on CFTR G_{Cl} was fully reversible. Following washout of glybenclamide, the magnitude of cAMP- and ATP-activated Cl^- diffusion potentials and conductances were restored to levels prior to the application of the inhibitor. Preparation and perfusion solutions as in Fig. 5.

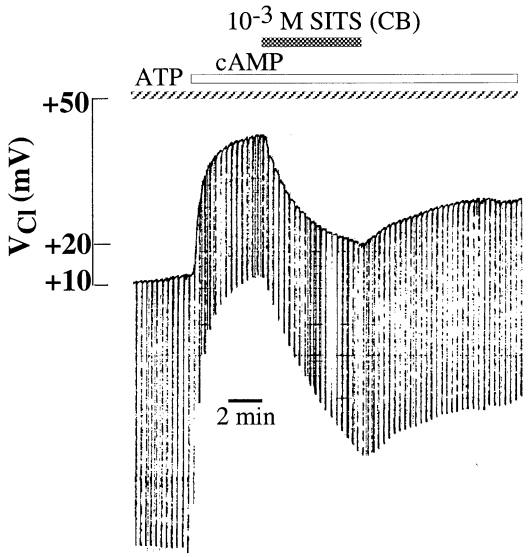
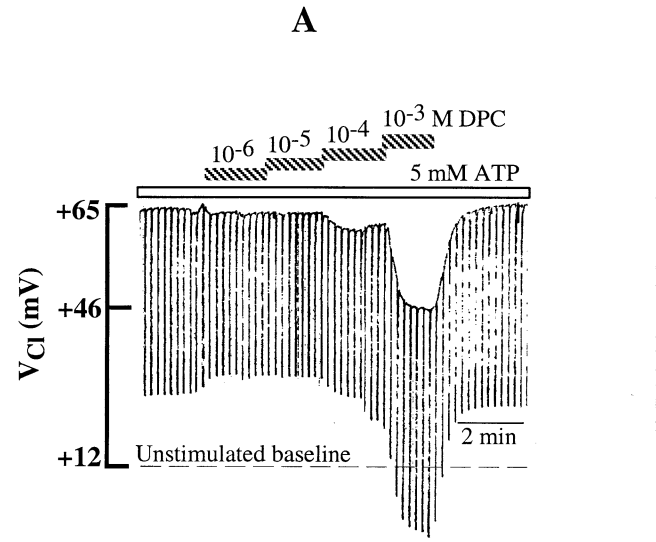


Fig. 9. The effect of SITS on activated CFTR G_{Cl}. CFTR G_{Cl} in the apical membranes of basilaterally permeabilized ducts was activated by cAMP and ATP in the cytoplasmic bath. Notice that application of 10^{-3} M SITS to the cytoplasmic bath almost completely inhibited CFTR G_{Cl}, which was partially reversible after washout of SITS.

inhibition of CFTR G_{Cl}. DIDS at 10^{-6} M partially inhibited CFTR G_{Cl} (ca. 10%). Even so, 10^{-3} M DIDS was required to obtain still less than complete blockage (86 ± 10%; Fig. 13).

Discussion

The human reabsorptive sweat duct presents a good opportunity to study the properties of the CFTR Cl^- channel in a native epithelial membrane. The apical membrane of the sweat duct is mainly comprised of ENaC and CFTR Cl^- channels [27, 32]. Likewise, the basilateral membrane is mainly comprised of Ba^{2+}-sensitive, Ca^{2+}-dependent K^+ channels (that provide a leakage pathway for excess K^+ accumulated during active transport of Na^+) and CFTR Cl^- channels [30, 33]. The simplicity of this model system allows characterization of potential pharmacological agents on ion channel targets (Na^+, K^+ and Cl^- conductances) in the basilateral and apical membranes by following the transepithelial potentials and conductances. In this study, after finding poor effects of these inhibitors on the electrical properties of intact



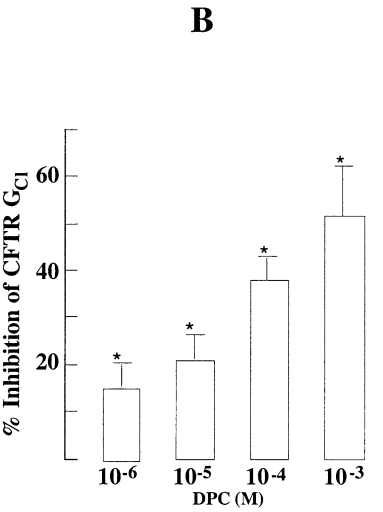
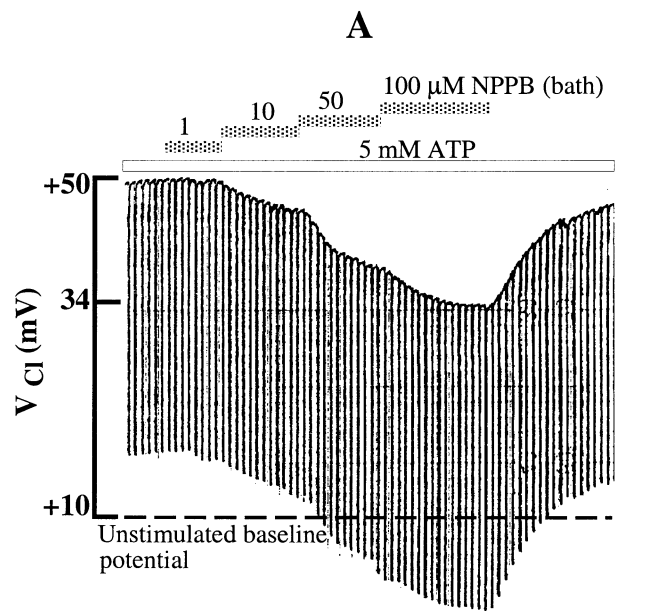


Fig. 10. Dose response of DPC on stably phosphorylated CFTR. (*A*) Application of DPC after phosphorylation of CFTR with ATP-γ-S partially inhibited CFTR G_{Cl}. The inhibitory effect of DPC was fully reversible and comparable to that observed before stable phosphorylation of CFTR, indicating that DPC did not affect CFTR through its phosphorylation or dephosphorylation. (*B*) Even at 10^{-6} M, DPC in cytoplasm caused a slight but significant decrease of CFTR G_{Cl} after stable phosphorylation of CFTR. The results were standardized to reflect percent inhibition, assuming that the CFTR G_{Cl} is 100 and zero in the presence and absence of ATP in the cytoplasmic bath, respectively. The values of CFTR G_{Cl} reflect the mean ± SE obtained from a minimum of 6 ducts. *Indicates statistical significance of difference between the percent inhibition of activated CFTR under the experimental conditions (in the presence of the inhibitor) and under control conditions (in the absence of the inhibitor; i.e., 0% inhibition). Notice that even at the highest concentration of DPC (10^{-3} M) only 50% inhibition of CFTR G_{Cl} occurred. Preparation and perfusion solutions as in Fig. 5.



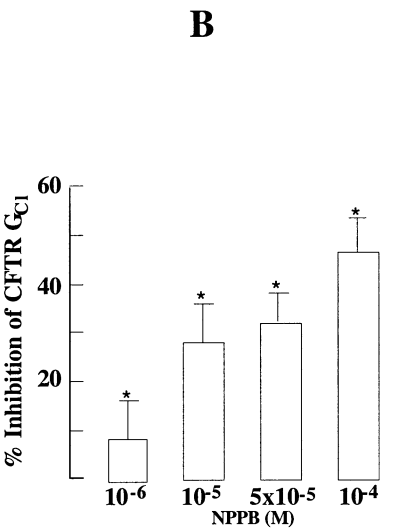


Fig. 11. Effect of NPPB on stably phosphorylated CFTR G_{Cl}. (*A*) In this tracing, CFTR was stably phosphorylated in the presence of cAMP + ATP-γ-S + PIC, as described in the Methods. The effect of increasing concentrations of NPPB in the cytoplasm is shown on ATP-activated CFTR G_{Cl}. (*B*) Summary of the data collected from experiments similar to those shown in panel *A*. Like DPC, NPPB in the cytoplasm caused a small, but detectable inhibition (10%) of CFTR G_{Cl} at the lowest concentration tested (10^{-6} M), but even at a concentration of 100 μM, NPPB only inhibited ~ 45% of CFTR G_{Cl}. The values of CFTR G_{Cl} reflect the mean ± SE obtained from a minimum of three ducts at each concentration. Statistics as in Fig. 10. Preparation and perfusion solutions as in Fig. 5.

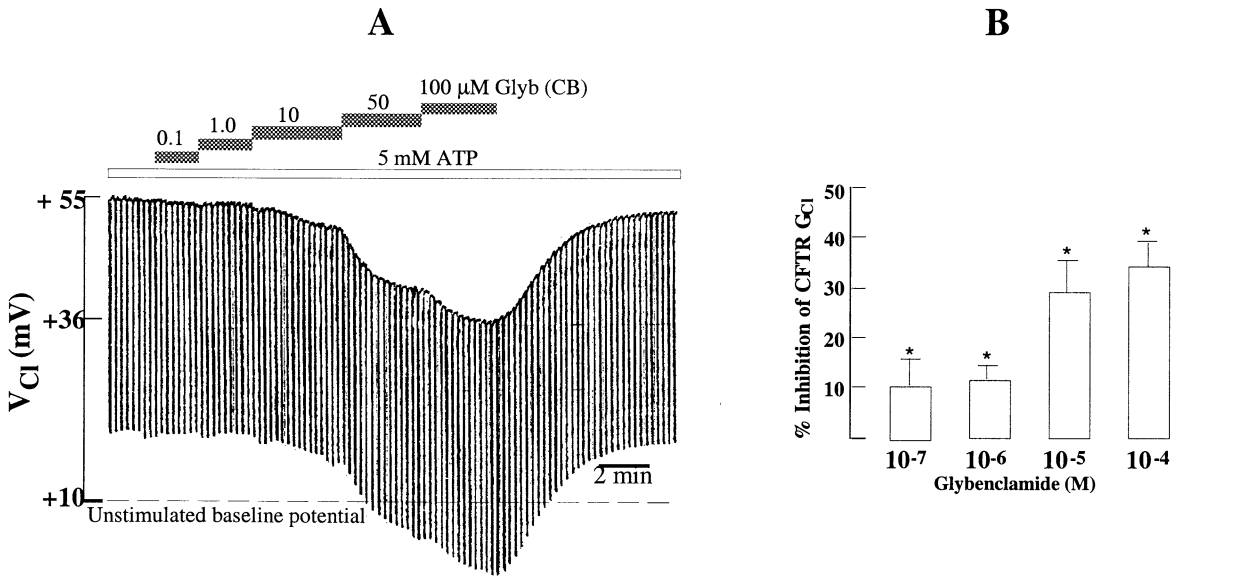


Fig. 12. Effect of glybenclamide on stably phosphorylated CFTR. (*A*) The effectiveness of stable phosphorylation of CFTR was confirmed by subsequent activation of CFTR G_{Cl} by application of 5 mM ATP alone without cAMP. The effect of increasing concentrations of glybenclamide on ATP-activated CFTR G_{Cl} is shown. Glybenclamide (10^{-7} M) caused a very small, but significant, inhibition of CFTR G_{Cl} of ~10%, but even at the limit of its solubility (10^{-4} M), glybenclamide inhibited only 35% of the activated CFTR G_{Cl}. (*B*) Summary of data collected from similar experiments as shown in panel *A*. The values of CFTR G_{Cl} reflect the mean ± SE obtained from a minimum of three ducts. Preparation and perfusion solutions as in Fig. 5. Statistics as in Fig. 10.

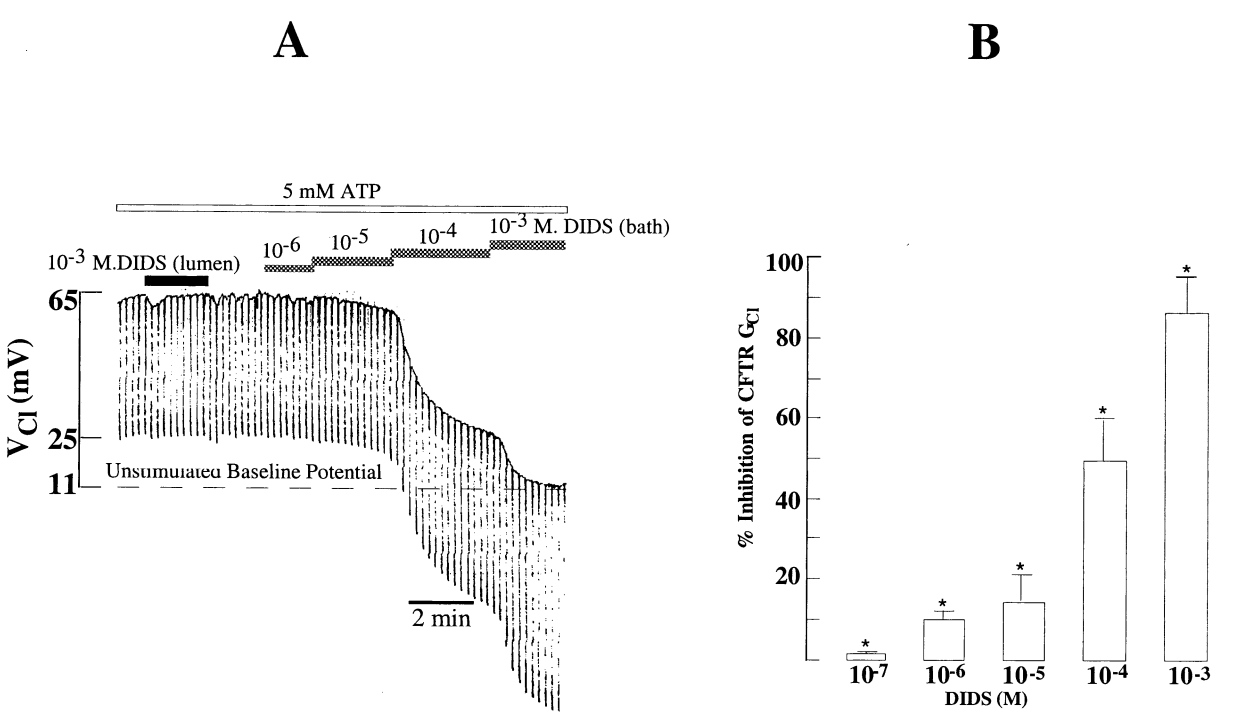


Fig. 13. Effect of DIDS on stably phosphorylated CFTR G_{Cl}. (*A*) Luminal application of DIDS had no effect on CFTR G_{Cl}, but increasing cytoplasmic concentrations of DIDS to 10^{-3} M lowered G_{Cl} almost to unstimulated levels. Furthermore, the effect of cytosolic DIDS on CFTR G_{Cl} was irreversible (*not shown*). (*B*) Summary of the data collected from similar experiments as shown in panel *A*. At ~10^{-3} M, cytosolic DIDS inhibited about 85% of the activated CFTR. The values of CFTR G_{Cl} are the mean ± SE obtained from a minimum of three ducts. Preparation and perfusion solutions as in Fig. 5. Statistics as in Fig. 10.

ducts, we simplified the system to selectively study the effect of the inhibitors on Cl^- conductances by permeabilizing the basilateral membrane and creating an ion gradient that selected for Cl^- [26, 28, 34].

Poor Inhibition of CFTR G_{Cl} from the Extracytosolic Side

Glybenclamide, DPC and NPPB have been shown to block CFTR Cl^- channels when applied to the extracellular surface in *ex vivo* model systems [12, 39]. We also showed that the loop diuretic bumetanide blocks CFTR when applied to either the apical or the basilateral surface of the sweat duct [34]. Using intact nonpermeabilized microperfused sweat ducts, we determined the acute effects of luminal and serosal application of DPC, NPPB, glybenclamide, SITS, DIDS and DNDS on CFTR. As shown in Figs. 1–3, except for a slight effect with DIDS from the serosal side, none of these inhibitors had any detectable effect on the electrical properties of sweat duct that would indicate inhibition of CFTR G_{Cl}. Loss of CFTR G_{Cl} in cystic fibrosis or block of CFTR with bumetanide markedly hyperpolarizes V_t and decreases transepithelial conductance [31, 34]. We expected that pharmacologically blocking G_{Cl} (CFTR) in the duct would produce similar hyperpolarizing effects from these inhibitors if they were effective from the extracellular surfaces. The apparent anomalous effect of serosal DIDS (1 mM), which caused a small hyperpolarization of V_t and a small increase in transepithelial resistance (Fig. 3), suggests that this stilbene may have a small inhibitory effect on the basilateral membrane G_{Cl}. However, this effect might be due to DIDS diffusing across the basilateral membrane and affecting the cytoplasmic surface of the apical membrane. The effect is probably not due to inhibition of a Cl^-/HCO_3^- exchanger or K^+ conductance because 1) we used nominally HCO_3^- -free medium, and 2) inhibition of K^+ conductance should have depolarized and not hyperpolarized V_t (Fig. 3). The lack of effect from either surface (with the exception of a possible, small inhibition of K^+ conductance by NPPB, and a very small inhibition of basilateral G_{Cl} by DIDS from the serosal side) indicates that these compounds are not effective in blocking CFTR from the extracellular surface. Since these inhibitors do block CFTR when applied to the cytoplasmic side in the permeabilized duct (*see* the discussion below), the apparent lack of effect of the lipophilic compounds (DPC, NPPB and glybenclamide) when applied to the luminal surface suggests that the inhibitors did not reach the cytoplasmic side in sufficiently high concentrations due to short duration of application.

The Effect of Inhibitors in the Cytoplasm

To test whether the inhibitors affect CFTR G_{Cl} when applied to the cytoplasmic surface of the apical membranes, we permeabilized the basilateral membrane with α-toxin. We activated CFTR G_{Cl} in the presence of cAMP and ATP, as described earlier. Application of DPC, NPPB, glybenclamide, SITS, DIDS and DNDS inhibited CFTR in a dose-dependent manner as indicated by decreased Cl^- diffusion potentials and CFTR G_{Cl} (Figs. 5–9). None of the inhibitors completely blocked G_{Cl} even at the highest possible concentrations, even though the best inhibitor, DIDS (1 mM) (Fig. 13) blocked ~85% of G_{Cl}. Since glybenclamide was shown to block the open CFTR channel [36], we tested the effect of glybenclamide both before and after activating CFTR with cAMP and ATP and found little difference in the magnitude of its maximal inhibition in either case (Figs. 7 and 8). These results suggest that the glybenclamide inhibition is independent of activation state of CFTR in the native sweat duct.

Direct Inhibition of CFTR

Since the G_{Cl} of the apical membrane of the sweat duct appears to be due exclusively to CFTR [26, 28, 34] and since we have arranged the experimental conditions to predominantly (if not exclusively) reflect only the Cl^- conductance properties of the apical membrane in permeabilized ducts, we take the inhibitor effects to be due to inhibition of CFTR channel activity. However, at least some of the inhibitors studied here have been shown to have multiple effects on cellular functions so that inhibition of CFTR function might be indirect. Examples of indirect effects of the inhibitors include inhibition of: a) mitochondrial ATP synthesis by uncoupling oxidative phosphorylation (NPPB) [18, 19], b) cyclooxigenase (DPC), and c) protein kinase A (glybenclamide) [36]. Therefore, it is possible that some of these inhibitors might act indirectly. In order to discount this possibility, we tested the effect of the inhibitors on stably phosphorylated CFTR as described [27, 32]. As previously reported, thio-ester-phosphorylated CFTR is stable and can be activated by ATP alone; no cAMP is required in the cytoplasm to activate protein kinase A since CTFR is and remains phosphorylated. Under these conditions, if an inhibitor reduces CFTR activity, its effect is most likely due to a direct effect on CFTR and not due to an indirect effect of inhibiting a protein kinase or activating a phosphatase. Moreover, since ATP is present in the cytoplasmic bath, the inhibitory effects cannot be attributed to metabolic poisoning or reduced ATP levels. Thus, we surmise that all the inhibitors tested (DPC, NPPB, glybenclamide and DIDS) probably blocked the phosphorylated, ATP-activated form of CFTR by direct interaction (Figs. 10–13).

In contrast to the irreversible effects of SITS and DIDS, which is consistent with their effects on the anion exchanger in the membranes of the red blood cells, the effect of DNDS was fully reversible (Figs. 5

and 9). The apparent largely irreversible binding of DIDS and SITS is attributed to the presence of the isothiocyano structure (-NCS) [7]. However, we incidentally found that raising cytoplasmic pH from 6.8 to 8.5 reversed the inhibition by SITS and DIDS. These results appear similar to the observations on ATP-sensitive K^+ channels [13]. However, this may not be taken as a reflection of structural similarities between CFTR and K_{ATP} channels, particularly in view of the fact that the effective concentration of glybenclamide is an order of magnitude higher than those required to inhibit K_{ATP} channels. Taken together with the fact that DIDS inhibits Cl^-/HCO_3^- exchangers only from the extracellular side and that it has no effect on ENaC from either cytosolic or luminal side (Fig. 4), DIDS may be the most effective inhibitor available for blocking CFTR anion channel function, at least for Cl^- absorption in a native tissue.

In addition, there is a significant discrepancy in the ability of several of these blockers to inhibit CFTR in different systems. 100 μM glybenclamide inhibited CFTR Cl^- currents by 90% in NIH 3T3 fibroblasts [38] but caused only ~34% inhibition of CFTR G_{Cl} in the permeabilized sweat ducts at similar concentration. While the exact cause(s) of such discrepancies are not clear at this time, it is possible that the differences in cytosolic composition such as ATP, ADP, pH and even membrane voltage may determine the effective inhibitor concentration [20, 38, 39]. For example, inhibition of CFTR by DPC appears to be voltage dependent [20] and pharmacological effects of sulfonylureas are not only tissue dependent but also influenced by ATP and ADP [38, 39].

Possible Effects on Other Transport Processes

DPC, NPPB and glybenclamide have been widely used as blockers of CFTR Cl^- channels [2, 6, 9, 20, 36–39]. But these inhibitors also block other cation channels including basilateral K^+ channels (NPPB) [15, 35], nonselective cation channels (DPC) [24] and ATP-sensitive K^+ channels (glybenclamide) [39].

G_K

NPPB caused a small but significant depolarization of V_t with a concomitant decrease of G_t (Fig. 2). These results are consistent with the inhibition of a G_K in the basilateral membrane since we have previously shown that inhibiting basilateral G_K by Ba^{2+} depolarizes both the basilateral membrane potential and V_t [30], and that the only other major ion conductance in the basilateral membrane is CFTR G_{Cl} [29, 33]. Inhibition of apical CFTR should have hyperpolarized V_t [29]. Inhibition of CFTR G_{Cl} in the basilateral membrane in cystic fibrosis ducts hyperpolarizes both the basilateral membrane and V_t [29]. The increased transepithelial resistance and decreased transepithelial potential are consistent only with inhibition of G_K in the basilateral membrane. Since NPPB does not block ENaC when applied to the cytosolic side in basilaterally permeabilized ducts (*results not shown*), the increase in transepithelial resistance and depolarization of V_t cannot be attributed to inhibition of G_{Na} in the apical membrane. Therefore, the results suggest that NPPB inhibits a G_K in the basilateral membrane, as reported for basilateral G_K in frog cornea [35].

Conclusions

The effects of DPC, NPPB, glybenclamide, SITS, DIDS and DNDS have some specificity for inhibiting CFTR because all the compounds 1) acted only from the cytosolic side, 2) showed dose dependent inhibition, 3) appeared to inhibit CFTR G_{Cl} independently of metabolic and phosphorylation effects, and 4) showed little, if any, effects on other transport functions. However, since these compounds are known to affect other Cl^- channels [3, 11, 16, 22] anion exchangers [6] and Cl^- co-transporters, caution must be exercised when they are used to specifically block CFTR G_{Cl}. Although DIDS appears to be the most effective blocker of CFTR in this tissue, none of the inhibitors completely blocked CFTR even at their limits of solubility. More effective and selective anion channel blockers are needed.

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