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

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Received: 20 December 2001/Revised: 15 May 2002

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 basally permeabilized native sweat duct epithelium. All of these inhibitors blocked CFTR in a dose-dependent manner from the cytoplasmic side of the basally 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 \( \sim 50\% \) 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.

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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 aryl-

aminobenzoates (DPC, NPPB), sulfonylurea (gly-
benclamide), and disulfonic stilbenes (DIDS, SITS, DNDS) in the freshly isolated, microperfused human sweat duct epithelium. Using intact and basolaterally α-toxin-permeabilized ducts we determined the sid-
edness 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 con-
sent. The isolated glands were transferred to a cuvette with Ringer’s solution cooled to 3\(^°\)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 solu-
tion containing 140 mm KGlue (potassium gluconate) and 5 mm ATP supplied to the basolateral surface of the microperfused sweat duct for 15 to 30 minutes. As described earlier [26], α-toxin effectively removes the basolateral 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 (co-
factors 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 basolateral 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 Elec-
tro-medical 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\(_h\)) with respect to the contraluminal bath and as a cannula for perfusing the lumen of the duct with selected solutions. V\(_h\) was monitored continuously using one channel of a WPI-700 dual electrometer referenced to the contraluminal bath. Transepithelial conductance (G\(_{Cl}\)) 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 basolateral 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 KGlue (bath); 150 mm NaCl (lumen)]. Under these conditions, V\(_h\) and G\(_{Cl}\) can be regarded as closely reflecting V\(_{Cl}\) and G\(_{Cl}\), respectively.

**Solutions**

The luminal perfusion Ringer’s solutions adjusted to pH 7.4 con-
tained (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\(_c\), (145), gluconate (Glu 140); PO\(_4\), 3.5; MgSO\(_4\), 1.2; and Ca\(^{2+}\), 0.26, buff-
ered with EGTA 2.0 mm to 80 mm 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 AMP (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\(^{-3}\) 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 inhibi-

tors: diphenylamine-2-carboxylate (DPC; 10\(^{-8}\) to 10\(^{-3}\) m), 5-nitro-

2-(3-phenylpropylamino) benzoic acid (NPPB; 10\(^{-6}\) to 10\(^{-3}\) 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 Glyben-

clamide (stock solution contained 0.05 m in ethanol) were prepared from previously mixed stock solutions. DIDS and SITS were