Substrates of multidrug resistance-associated proteins block the cystic fibrosis transmembrane conductance regulator chloride channel

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1   The effects of physiological substrates of multidrug resistance-associated proteins (MRPs) on cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel currents were examined using patch clamp recording from CFTR-transfected mammalian cell lines.

2   Two MRP substrates, taurolithocholate-3-sulphate (TLCS) and β-estradiol 17-(β-D-glucuronide) ($E_2 17\beta G$) caused a voltage-dependent block of macroscopic CFTR Cl^- currents when applied to the intracellular face of excised membrane patches, with mean apparent dissociation constants (K_Ds) of 96 ± 10 and 563 ± 103 μM (at 0 mV) respectively. The unconjugated bile salts taurocholate and cholate were also effective CFTR channel blockers under these conditions, with K_Ds of 453 ± 44 and 3760 ± 710 μM (at 0 mV) respectively.

3   Reducing the extracellular Cl^- concentration from 154 to 20 mM decreased the K_D for block intracellular TLCS to 54 ± 1 μM, and also significantly reduced the voltage dependence of block, by suggesting that TLCS blocks Cl^- permeation through CFTR by binding within the channel pore.

4   Intracellular TLCS reduced the apparent amplitude of CFTR single channel currents, suggesting that the duration of block is very fast compared to the gating of the channel.

5   The apparent affinity of block by TLCs is comparable to that of other well-known CFTR channel blockers, suggesting that MRP substrates may comprise a novel class of probes of the CFTR channel pore.

6   These results also suggest that the related proteins CFTR and MRP may share a structurally similar anion binding site at the cytoplasmic face of the membrane.

Keywords: CFTR; chloride channel; multidrug resistance-associated protein; ABC protein; channel block; sulphonylurea; cystic fibrosis

Abbreviations: ABC, ATP-binding cassette; BHK, baby hamster kidney; BSEP, bile salt export pump; cBAT, canalicular bile acid transporter; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; cMOAT, canalicular multispecific organic anion transporter; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; DNDs, 4,4′-dinitrostilbene-2,2′-disulphonic acid; DPC, diphenylamine-2-carboxylate; $E_2 17\beta G$, β-estradiol 17-(β-D-glucuronide); GSH, reduced glutathione; GSSG, oxidized glutathione; MRP, multidrug resistance-associated protein; PKA, protein kinase A catalytic subunit; TLCS, taurolithocholate-3-sulphate; TM, transmembrane region; z', apparent valence

Introduction

Cystic fibrosis is caused by mutations in a single gene, that encoding the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a member of the ATP-binding cassette (ABC) family of membrane proteins, most members of which are thought to form ATP-dependent pumps of a wide range of substances (Higgins, 1995). In contrast, CFTR is a phosphorylation- and ATP-dependent ion channel which mediates the passive electrodiffusion of Cl^- ions (Gadsby *et al.*, 1995; Hanrahan *et al.*, 1995). However, recent evidence has suggested some degree of functional similarity between CFTR and the closely related ABC proteins, multidrug resistance-associated protein (MRP) and canalicular multispecific organic anion transporter (cMOAT; also known as MRP2), both of which function as ATP-dependent pumps of a broad range of large intracellular organic anions (see below). We have shown that the CFTR Cl^- channel is permeable to a number of large organic anions from the cytoplasmic side of the membrane (Linsdell & Hanrahan, 1998a), including the MRP- and

cMOAT-substrate oxidized glutathione (GSSG; Linsdell & Hanrahan, 1998b). Such large anions are also able to block Cl^- permeation through CFTR when present in the intracellular solution (Linsdell & Hanrahan, 1996a; 1998b), suggesting that large anions and Cl^- ions pass through the same pore in CFTR, but that large anions have a longer residency time within the pore and therefore slow the overall rate of permeation.

MRP and cMOAT show similar yet broad substrate specificities, both transporting a diverse range of glutathione, glucuronide, and sulphate conjugates (Oude Elferink *et al.*, 1995; Keppler *et al.*, 1997). Physiological substrates for these transporters may include cysteinyl leukotrienes such as leukotriene C_4 (LTC_4; Leier *et al.*, 1994; Oude Elferink *et al.*, 1995), conjugated bile salts such as taurolithocholate-3-sulphate (TLCS; Oude Elferink *et al.*, 1995; Jedlitschky *et al.*, 1996) and conjugated steroids such as β-estradiol 17-(β-D-glucuronide) ($E_2 17\beta G$; Loe *et al.*, 1996; Keppler *et al.*, 1997).

We have investigated the effects of two physiological MRP/cMOAT substrates on CFTR Cl^- channel activity. The high affinity MRP/cMOAT substrates TLCS and $E_2 17\beta G$ both caused a voltage-dependent block of macroscopic CFTR Cl^-

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currents when applied to the cytoplasmic face of the membrane. The unconjugated bile salts taurocholate and cholate were also effective voltage-dependent blockers. Intracellular TLCS caused a voltage-dependent reduction in unitary CFTR Cl$^-$ current, suggesting a blocking mechanism with kinetics much faster than those of channel opening and closing. These results identify MRP/cMOAT substrates as a novel class of probes of the CFTR Cl$^-$ channel, and suggest some structural similarity between CFTR and other related ABC proteins at the cytoplasmic face of the membrane.

Methods

CFTR macroscopic and single channel current recordings were both made using the excised, inside-out configuration of the patch clamp technique, as described previously (Linsdell & Hanrahan, 1996b; 1998a; Linsdell et al., 1997). Baby hamster kidney (BHK) cells were used for macroscopic recordings and Chinese hamster ovary (CHO) cells for single channel recordings (Linsdell & Hanrahan, 1998a). Briefly, channels were activated by exposure of the cytoplasmic face of the patch to 40–180 nM protein kinase A catalytic subunit (PKA; prepared in the laboratory of Dr M. P. Walsh, University of Calgary, Alberta, Canada, as described previously; Tabcharani et al., 1991) plus 1 mM MgATP (Sigma, St Louis, MO, U.S.A.). Macroscopic current-voltage (I-V) relationships were

constructed using depolarizing ramp protocols and have had the background (leak) current recorded before the addition of PKA subtracted digitally, as described previously (Linsdell & Hanrahan, 1996b; 1998a). Current traces were filtered at 100 Hz (for macroscopic currents, I) or 50 Hz (for single channel currents, i) using an 8-pole Bessel filter, and digitized at 250 Hz. Macroscopic currents were analysed using pCLAMP6 computer software (Axon Instruments, Foster City, CA, U.S.A.), while single channel currents were analysed using custom-written pCLAMP-compatible DRSCAN software (Hanrahan et al., 1998).

All recording solutions contained (mM) NaCl 150, MgCl$_2$ 2, TES (pH 7.4) 10 except where the effects of reducing extracellular Cl$^-$ concentration were studied (Figure 4), when the pipette solution contained (mM) NaCl 20, Na·gluconate 134, Mg(OH)$_2$ 2, TES (pH 7.4) 10. Channel blockers (all obtained from Sigma, except glibenclamide (glyburide; Calbiochem, La Jolla, CA, U.S.A.), 4,4'-dinitrostilbene-2,2'-disulphonic acid (DNDS; Pfaltz & Bauer, Waterbury, CT, U.S.A.) and diphenylamine-2-carboxylate (DPC; N-phenylanthranilic acid; Fluka Chemika, Buchs, Switzerland)) were added to the intracellular solution from concentrated stocks made up in recording solution and stored frozen until just before the experiment.

The effects of CFTR channel blockers were analysed according to the Woodhull (1973) model of voltage dependent block:
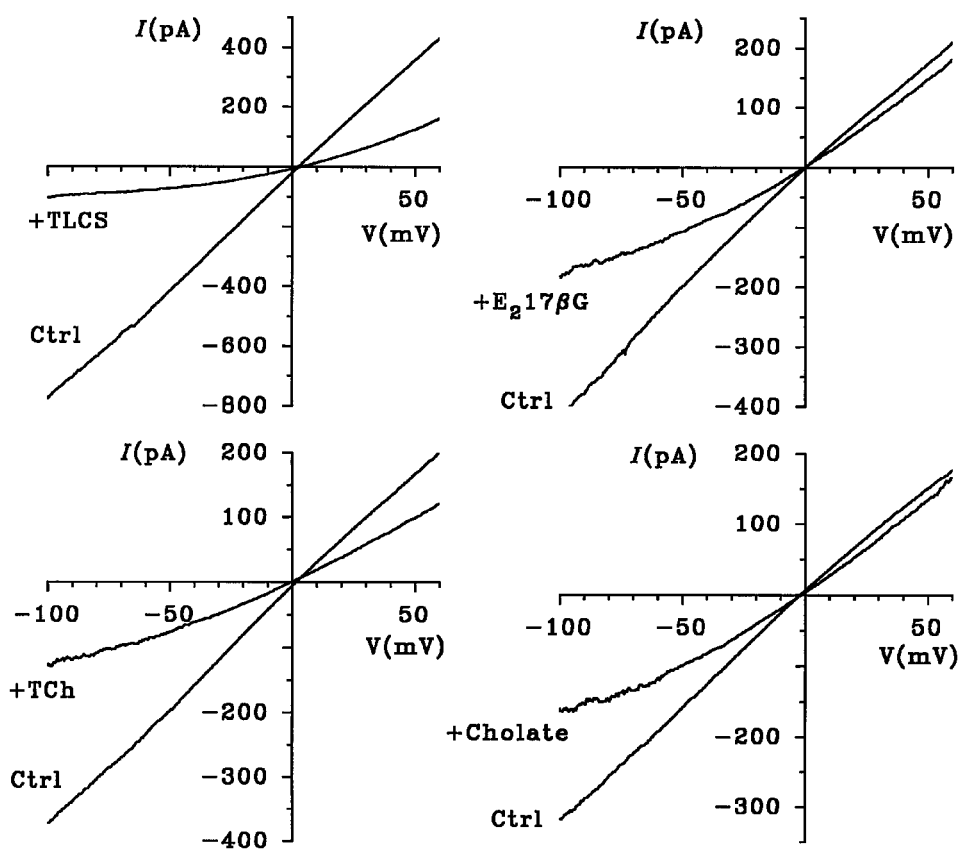

Figure 1 Voltage-dependent block of macroscopic CFTR Cl$^-$ currents by MRP/cMOAT substrates. Each panel shows an example macroscopic I-V relationship recorded before (ctrl.) and immediately after addition of TLCS (200 μM), E$_2$17βG (200 μM), taurocholate (TCh; 500 μM) or cholate (1 mM) to the intracellular solution. Experiments with different blockers were carried out on different patches. CFTR currents were activated by addition of PKA in the presence of MgATP, and reached steady-state levels 2–5 min after addition of PKA. Patches were held at 0 mV and depolarizing voltage ramps applied at a rate of 100 mV s^{-1}. Current-voltage relationships were leak subtracted as described in Methods.

$$I/I_0 = K_D(V)/(K_D(V) + [B]) \qquad (1)$$

where I is the amplitude of the current remaining in the presence of blocker, I_0 is the control, unblocked current amplitude, $[B]$ is the blocker concentration, and $K_D(V)$ is the voltage dependent dissociation constant, the voltage dependence of which is given by:

$$K_D(V) = K_D(0) \exp(-z' \, VF/RT) \qquad (2)$$

where $K_D(0)$ is the dissociation constant at 0 mV, z' is the apparent valence of the blocking ion, that is the real valence (-2 for TLCS and DNDS, -1 for all other blockers studied here) multiplied by δ, the fraction of the membrane electric field experienced by the blocking ion, V is the membrane potential, and F, R and T have their normal thermodynamic meanings.

Experiments were carried out at room temperature, 21–23°C. Mean values are presented as mean$\pm$s.e.mean. For graphical presentation of mean values, error bars represent $\pm$ s.e.mean, where this is larger than the size of the symbol.

Results

The effects of the MRP/cMOAT substrates TLCS and $E_217\beta G$ on macroscopic CFTR Cl$^-$ currents were examined in inside-out membrane patches excised from BHK cells stably expressing CFTR (Linsdell & Hanrahan, 1996b; see Methods). As shown in Figure 1, the addition of TLCS (200 μM) or $E_217\beta G$ (200 μM) to the intracellular solution caused a marked reduction in CFTR Cl$^-$ current. In both cases the block was clearly voltage-dependent, with the reduction in current amplitude being strongest at negative voltages, as would be expected for block of the channel pore within the transmembrane electric field by a negatively charged molecule acting from the inside. Block occurred immediately on application of either TLCS or $E_217\beta G$, and in both cases block was readily

reversible on washing (not shown). The unconjugated bile salts taurocholate and cholate were also effective voltage dependent blockers of CFTR Cl$^-$ current when added to the intracellular solution (Figure 1). Although these bile salts are thought not to be transported by MRP or cMOAT in the unconjugated form, they may inhibit MRP- or cMOAT-mediated transport of other substances (Holló et al., 1996; Loe et al., 1996; Saxena & Henderson, 1996). The blocking effects of all of these substances was analysed using the Woodhull (1973) model (see Methods); results are summarized in Table 1. The chemical structures of these substances are given in Figure 2.

Of those substances tested in Figure 1, TLCS was the highest affinity blocker of CFTR Cl$^-$ current (Table 1). The concentration dependence of block of macroscopic CFTR

Table 1 Blockers of macroscopic CFTR Cl$^-$ current in inside-out BHK cell patches

| Blocker	$K_d(0)$ (μM)	$-z'$	n
Glibenclamide	67 ± 9	0.37 ± 0.03	6
DIDS*	77 ± 15	0.16 ± 0.02	4
TLCS	96 ± 10	0.37 ± 0.05	16
DNDS*	162 ± 16	0.34 ± 0.03	7
Taurocholate	453 ± 44	0.16 ± 0.01	5
DPC	461 ± 51	0.27 ± 0.05	5
$E_217\beta G$	563 ± 103	0.27 ± 0.03	5
Cholate	3760 ± 710	0.29 ± 0.05	4
GSH**	15800 ± 3700	0.11 ± 0.02	5
Glucuronate	21600 ± 900	0.01 ± 0.03	4
GSSG**	23700 ± 2900	0.02 ± 0.03	4
Sulphate	53200 ± 10200	0.00 ± 0.03	4

*From Linsdell & Hanrahan (1996b). **From Linsdell & Hanrahan (1998b). $K_d(0)$ and z' were calculated for different CFTR channel blockers under similar conditions as described in Methods. Our results for glibenclamide and DPC are similar to those previously reported by Sheppard & Robinson (1997) and McCarty et al. (1993) respectively. GSH, reduced glutathione; GSSG, oxidized glutathione.
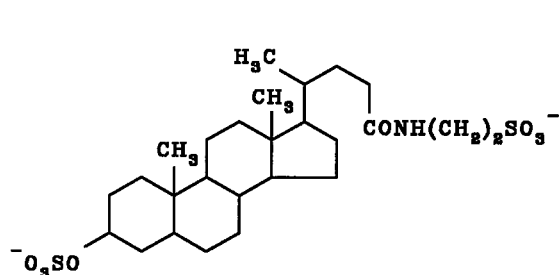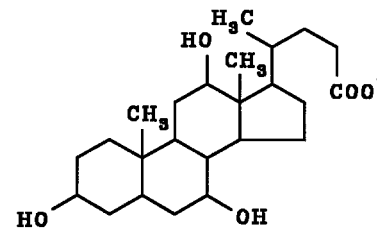

Taurolithocholate–3–sulphate (TLCS)
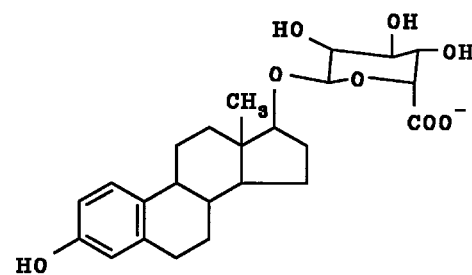
β–Estradiol 17–(β–D–Glucuronide) ($E_217\beta G$)
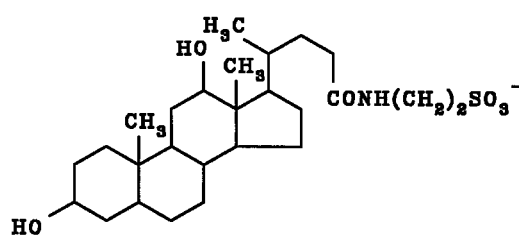
Taurocholate

Cholate

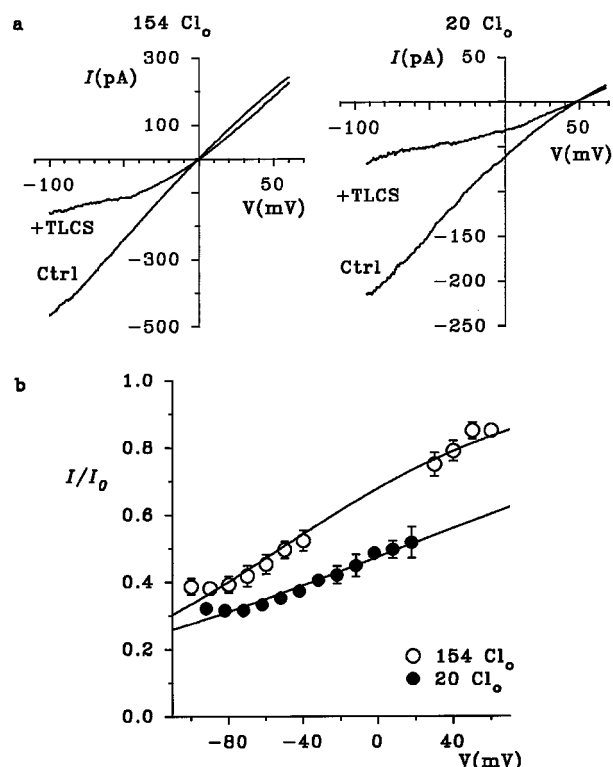**Figure 2** Chemical structures of the blockers used in Figure 1.

Figure 4 Dependence of block by intracellular TLCS on extra-cellular Cl^- concentration. (a) Effects of 60 μM TLCS on macroscopic DFTR currents recorded with 154 mM (left) or 20 mM (right) Cl^- in the pipette (extracellular) solution (Cl_o). (b) Mean fraction of control current remaining (I/I_0) following addition of 60 μM TLCS as a function of voltage. The data have been fitted by equation 1, giving a $K_D(0)$ of 109 μM and a $-z'$ of 0.36 with 154 mM Cl^-, and a $K_D(0)$ of 54 μM and a $-z'$ of 0.22 with 20 mM Cl^-.

Figure 3 Concentration and voltage dependence of block by TLCS. (a) Mean fraction of control current remaining (I/I_0) following addition of different concentrations of TLCS, at different membrane potentials. $n = 3-4$ patches. The data at each voltage have been fitted by equation 1, as described in the text. (b) Dissociation constant, K_D, for TLCS block as a function of membrane potential. K_D was calculated from equation 1 as described in the text. The fitted straight line indicates a K_D of 93.2 μM at 0 mV at and apparent valency (z') of -0.35 for TLCS.

current by intracellular TLCS is described in Figure 3a. At each concentration studied, the mean fraction of control current remaining following addition of TLCS (I/I_0) was calculated from 3–4 patches at -100, -50 and $+50$ mV. At each potential, the data have been fitted by equation 1 (see Methods), suggesting that a single TLCS molecule blocks the channel with a K_D of 25.4 μM at -100 mV, 45.3 μM at -50 mV, and 186.8 μM at $+50$ mV. Similar analyses at other potentials allowed examination of the voltage dependence of block (Figure 3b). The fitted straight line gives a K_D of 93.2 μM at 0 mV, and its slope an apparent valency, z', of -0.35 for TLCS, according to equation 2 (see Methods).

The voltage dependence of block by MRP substrates suggests that these substances block CFTR Cl^- currents by binding within the pore. Consistent with this hypothesis, block by intracellular TLCS was enhanced by reducing the extracellular Cl^- concentration (Figure 4). With 154 mM Cl^- in the pipette solution, 60 μM TLCS blocked macroscopic currents with a mean $K_D(0)$ of 109 ± 16 μM and a $-z'$ of

0.36 ± 0.02 ($n = 6$); note that these values are similar to those obtained from the concentration dependence of block shown in Figure 3. Reducing external Cl^- concentration to 20 mM (by replacement with gluconate) significantly reduced both the $K_D(0)$ (to 54 ± 1 μM, $n = 5$) and also the $-z'$ (to 0.22 ± 0.01, $n = 5$) ($P < 0.01$ in both cases, Student's two-tailed t-test).

The effect of intracellular TLCS on CFTR single channel currents was examined in inside-out membrane patches excised from CHO cells stably expressing CFTR (Tabcharani et al., 1991; see Methods). Addition of 50 μM TLCS to the intracellular solution caused a voltage-dependent reduction in single channel current amplitude (Figure 5), suggesting that TLCS blocks the channel by a relatively fast mechanism, with individual blocking and unblocking events occurring too rapidly to resolve using patch clamp recording at the time resolution used. Intracellular TLCS did not appear to affect channel gating or open probability; indeed, the reduction in unitary amplitude was sufficient to account for the reduction in macroscopic current amplitude due to TLCS. Inclusion of 50 μM TLCS in the pipette solution has no discernible effect on CFTR single channel current amplitude or gating (not shown), suggesting that TLCS blocks the channel preferentially from its cytoplasmic end. A number of other anions have previously been shown to block CFTR only from the cytoplasmic side of the membrane (e.g. gluconate (Linsdell & Hanrahan, 1996a), disulphonic stilbenes (Linsdell & Hanrahan, 1996b), glibenclamide (Sheppard & Robinson, 1997)).
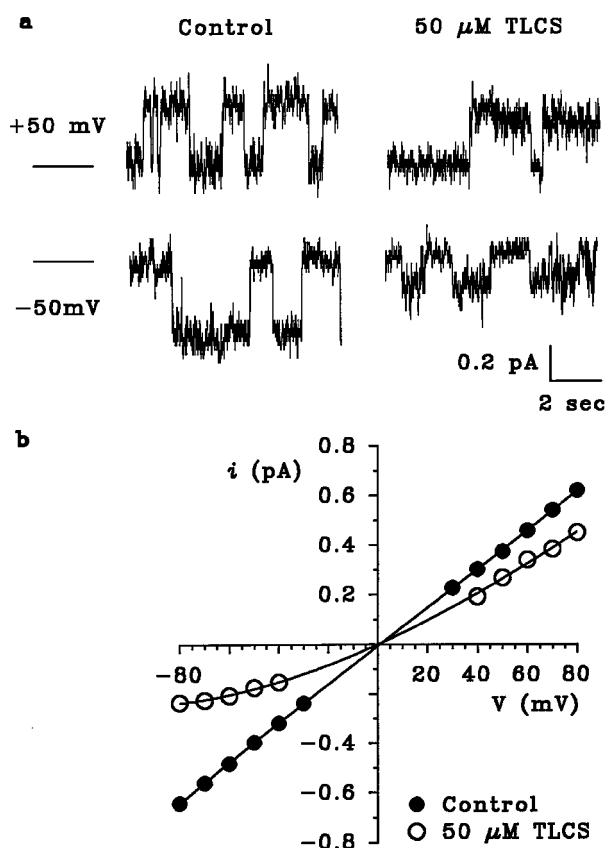
Figure 5 Block of unitary CFTR Cl^- currents by TLCS. (a) Single CFTR channel currents recorded at $+50$ mV (top) or -50 mV (bottom), before (control) and after addition of 50 μM TLCs to the intracellular solution. In each case the closed state of the channel is indicated by the line to the left. TLCS causes a voltage-dependent reduction in apparent single channel current amplitude, and effect which is illustrated more clearly by the mean single channel i-V relationships in (b). Mean of data from $3-18$ patches.

Figure 6 Voltage-dependent block of macroscopic CFTR Cl^- currents by different classes of CFTR channel blockers. As in Figure 1, example macroscopic I-V relationships were recorded before (ctrl) and immediately after addition of glibenclamide (Glib; 60 μM), DNDS (200 μM) or DPC (200 μM) to the intracellular solution. The structures of these chemically diverse blockers are also shown.

The apparent affinity with which TLCS and $E_2 17\beta G$ block CFTR Cl^- currents are much higher than those of unconjugated glutathione (Linsdell & Hanrahan, 1998b; Table 1), glucuronide, or sulphate (data not shown; Table 1), and are comparable to those previously described for other classes of CFTR channel blockers, such as the sulphonylureas glibenclamide and tolbutamide (Sheppard & Welsh, 1992; Schultz et al., 1996; Venglarik et al., 1996; Sheppard & Robinson, 1997), the arylaminobenzoates diphenylamine-2-carboxylate (DPC) and flufenamic acid (McCarty et al., 1993), and the disulphonic stilbenes 4,4'-dinitrostilbene-2,2'-disulphonic acid (DNDS) and 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS; Linsdell & Hanrahan, 1996b). The effects of intracellular glibenclamide, DNDS and DPC on macroscopic CFTR Cl^- currents in excised BHK cell patches are shown in Figure 6 for comparison. The affinity and voltage dependence of these blockers measured under the same conditions as in Figure 1 are summarized in Table 1. This table does not represent an exhaustive list of known CFTR channel blockers, but rather selected examples of different classes of blockers. For example, other arylaminobenzoates may block cardiac CFTR with a higher affinity than any blocker listed in Table 1 (Walsh & Wang, 1998). The chemical structures of glibenclamide, DNDS and DPC, as shown in Figure 6, indicate that they are unrelated to the novel CFTR channel blockers identified in the present study. However, MRP substrates and unconjugated bile sales are structurally related to oestrogens
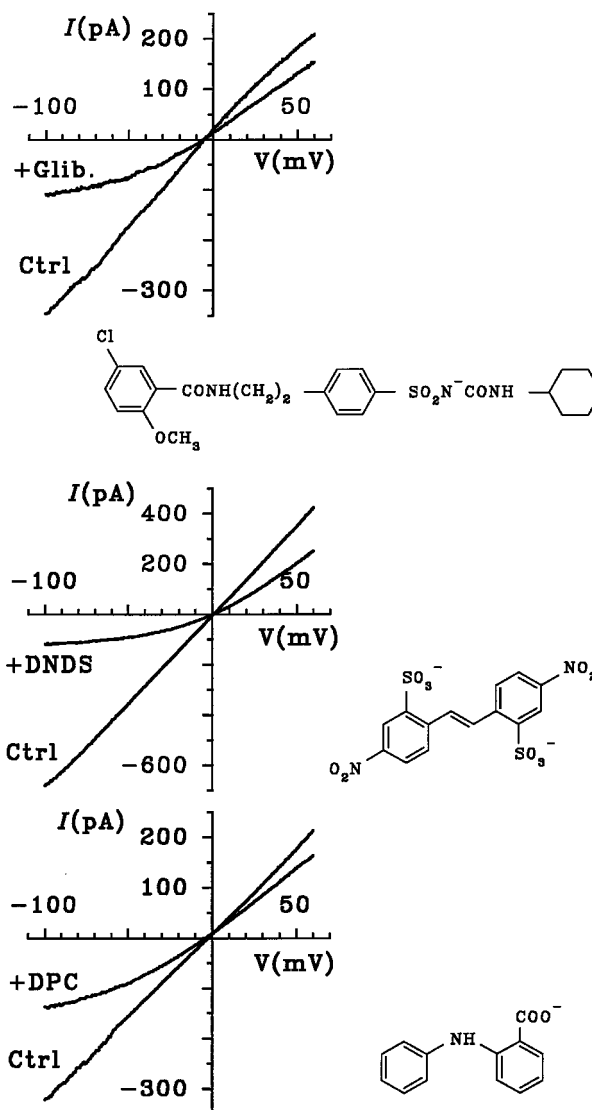
which have previously been reported to bind to and inhibit CFTR (Singh et al., 1994; 1995).

Discussion

Based on its structural similarity to other ABC proteins, CFTR was originally proposed to function as a pump (Hyde et al., 1990). However, subsequent experimental evidence proved that the primary (although perhaps not exclusive) function of CFTR is that of a Cl^- channel (see Gadsby et al., 1995; Hanrahan et al., 1995). Nevertheless, recent evidence has suggested that CFTR may share some functional similarities with other ABC proteins which do function as pumps, in particular its close structural relatives MRP and cMOAT (Lallemand et al., 1997; Linsdell & Hanrahan, 1998a, b). Our present results indicate that TLCS and $E_2 17\beta G$, both of which are high affinity substrates for both MRP- and cMOAT-mediated active transport, also act as blockers of the CFTR Cl^- channel. Whether these substances are actually permeant

in CFTR, as is the case for lower affinity anionic blockers such as gluconate and glutamate (Linsdell & Hanrahan, 1998a) and glutathione (Linsdell & Hanrahan, 1996b) is not addressed in the present study. Nevertheless, our results suggest some similarity between CFTR and other ABC proteins in terms of anion binding.

MPR and cMOAT transport a broad range of glutathione, glucuronide and sulphate conjugates (Oude Elferink *et al.*, 1995; Keppler *et al.*, 1997). We find that both the sulphate TLCS and the glucuronide $E_2 17\beta G$ cause voltage dependent block of Cl^- permeation in CFTR when applied to the cytoplasmic face of excised patches (Figure 1). In both cases the affinity of the blocking reaction (at 0 mV) was at least an order of magnitude higher than that associated with block by unconjugated glutathione, glucuronate or sulphate (Table 1).

The unconjugated bile salts taurocholate and cholate were also effective voltage dependent blockers of CFTR. Although these bile salts are thought not to be substrates for MRP- or cMOAT-mediated transport, they do inhibit transport of other substrates and thus probably interact with an anion binding site on the cytoplasmic face of these proteins (Holló *et al.*, 1996; Loe *et al.*, 1996; Saxena & Henderson, 1996). Unconjugated bile salts are transported by the hepatocanalicular protein known as canalicular bile acid transporter (cBAT; Müller & Jansen, 1997) or bile salt export pump (BSEP; Gerloff *et al.*, 1998). The gene encoding this protein was recently cloned from rat liver and named *spgp* (sister of P-glycoprotein; Gerloff *et al.*, 1998). Although the spgp protein shows closer homology to P-glycoprotein than to MRP or cMOAT, a yeast bile salt transporter (bile acid transporter, bat1p; Ortiz *et al.*, 1997) is homologous to both MRP and cMOAT. Thus a broad range of organic anions may bind to a homologous anion binding site on all these ABC proteins (CFTR, MRP, cMOAT, cBAT/BSEP), which contributes either to a cytoplasmic substrate binding site (in MRP, cMOAT and cBAT/BSEP) or to the Cl^- channel pore in CFTR. Similarly, substrates of P-glycoprotein inhibit an outwardly rectifying Cl^- channel in multidrug-resistant CHO cells overexpressing P-glycoprotein (Bear, 1994).

The affinity of CFTR channel block by MRP substrates, in particular TLCS, is comparable to that of other well known classes of CFTR channel blockers, namely sulphonylureas, disulphonic stilbenes, and arylaminobenzoates (Table 1),

although block by DPC (McCarty *et al.*, 1993) and glibenclamide (Schultz *et al.*, 1996; Sheppard & Robinson, 1997) appear kinetically slower than that by TLCS (Figure 5), with individual blocking events being resolved at the single channel level. MRP substrates may therefore comprise a novel class of probes with which to investigate the molecular architecture of the CFTR channel pore. The dependence of block by TLCS on voltage (Figures 1, 3 and 5) and on extracellular Cl^- concentration (Figure 4) are both consistent with binding within the channel pore. Previously, it has been shown that DPC interacts with CFTR residues serine 341 (located in the sixth transmembrane region, TM6) and threonine 1134 (in TM12) (McDonough *et al.*, 1994), and that DNDS interacts with TM6 residue arginine 347 (Linsdell & Hanrahan, 1996b), which suggests that these residues may contribute to intrapore anion binding sites. An interesting difference between the MRP substrates studied here and other CFTR channel blockers studied previously is that all of the blockers identified in the present study occur naturally within the body, raising the possibility that an endogenous cytoplasmic blocker may regulate CFTR conductance *in vivo* (Tabcharani *et al.*, 1991; Linsdell & Hanrahan, 1996a).

Block of the CFTR Cl^- channel is potentially of clinical as well as biophysical interest. Selective, high affinity CFTR channel blockers may be useful in the treatment of diarrhoea (Sheppard & Welsh, 1992). Furthermore, by analogy with other ion channels, it has been suggested that CFTR channel blockers might serve as lead compounds for the development of CFTR channel openers (Sheppard & Welsh, 1992; Schultz *et al.*, 1996), of great potential therapeutic relevance to cystic fibrosis. Anionic MRP/cMOAT substrates represent a novel class of CFTR channel blockers which might guide the development of clinically useful modulators of CFTR activity.

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