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IDENTIFICATION OF A SECOND BLOCKER BINDING SITE AT THE CYTOPLASMIC MOUTH OF THE CFTR CHLORIDE CHANNEL PORE

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Running Title Page

Running Title: Multiple Blocker Binding Sites in the CFTR Channel Pore

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Nonstandard Abbreviations Used: BHK, baby hamster kidney; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DNDS, 4,4'-dinitrostilbene-2,2'-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; PKA, protein kinase A catalytic subunit; PPi, pyrophosphate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; TLCS, taurolithocholate-3-sulfate.

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ABSTRACT

Chloride transport by the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel is inhibited by a broad range of substances that bind within a wide inner vestibule in the pore and physically occlude Cl^- permeation. Binding of many of these so-called open channel blockers involves electrostatic interactions with a positively charged lysine residue (K95) located in the pore. Here we use site-directed mutagenesis to identify a second blocker binding site located at the cytoplasmic mouth of the pore. Mutagenesis of a positively charged arginine at the cytoplasmic mouth of the pore, R303, leads to significant weakening of the blocking effects of suramin, a large negatively charged organic molecule. Apparent suramin affinity is correlated with the side chain charge at this position, consistent with an electrostatic interaction. In contrast, block by suramin is unaffected by mutagenesis of K95, suggesting that it does not approach close to this important pore-forming lysine residue. We propose that the CFTR pore inner vestibule contains two distinct blocker binding sites. Relatively small organic anions enter deeply into the pore to interact with K95, causing an open channel block that is sensitive to both the membrane potential and the extracellular Cl^- concentration. Larger anionic molecules can become lodged in the cytoplasmic mouth of the pore where they interact with R303, causing a distinct type of open channel block that is insensitive to membrane potential or extracellular Cl^- ions. The pore may narrow significantly between the locations of these two blocker binding sites.

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INTRODUCTION

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial cell Cl^- channel (Sheppard and Welsh, 1999; Kidd et al., 2004). Physiological regulation of CFTR activity occurs via stimulation by cAMP-dependent protein kinase A (Sheppard and Welsh, 1999; Kidd et al., 2004). CFTR activity is also subject to pharmacological modulation. A great deal of attention has been paid to the development of CFTR activators, since these substances hold great promise as pharmacological treatments for CF (Cai et al., 2004; Galietta and Moran, 2004). At the same time, it has been suggested that potent and selective CFTR inhibitors might prove useful in the treatment of secretory diarrhea and polycystic kidney disease (Galietta and Moran, 2004; Sheppard, 2004).

A number of different classes of CFTR inhibitors have been described (Cai et al., 2004), and these substances inhibit CFTR activity by two main mechanisms of action: inhibition of channel opening (gating inhibitors) and occlusion of the open channel pore (open channel blockers). Gating inhibitiors (also known as allosteric inhibitors; Cai et al., 2004) are presumed to act by interfering with the normal process of channel opening, most likely by interacting with the intracellular parts of the CFTR protein that control channel gating. Open channel blockers act by binding within the channel pore and physically occluding it, preventing the passage of Cl^- ions. A structurally diverse group of organic anions have been shown to inhibit CFTR function by an open channel block mechanism, including sulfonylureas, arylaminobenzoates, disulfonic stilbenes, indazoles, and conjugated bile salts (Schultz et al., 1999; Cai et al., 2004; Linsdell, 2005). The inhibitory effects of these different open channel blockers share a number of common features. First, they enter the pore from its intracellular end, meaning that they are often only effective when applied to the cytoplasmic side of the membrane (Linsdell and Hanrahan, 1996a;

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Sheppard and Robinson, 1997; Linsdell and Hanrahan, 1999). Secondly, their blocking effects are dependent on the membrane potential, being strongest at hyperpolarized voltages that would tend to drive negatively charged substances from the cytoplasm into the transmembrane electric field (McDonough et al., 1994; Linsdell and Hanrahan, 1996a; Sheppard and Robinson, 1997; Linsdell and Hanrahan, 1999; Gong et al., 2002b). Thirdly, their blocking effects are weakened by Cl^- ions on the *trans*- (extracellular) side of the membrane, suggesting that repulsive interactions between extracellular Cl^- ions and intracellular blocking ions occur within the channel pore (McDonough et al., 1994; Sheppard and Robinson, 1997; Linsdell and Hanrahan, 1999; Gong et al., 2002b). Recent work from our laboratory showed that these well-known open channel blockers also share a common molecular mechanism of action that involves electrostatic interactions with the positive charge of a lysine side chain (K95) within the channel pore (Linsdell, 2005).

In addition to conferring to the pore sensitivity to open channel block by organic anions, the positive charge of K95 acts to draw Cl^- ions into the pore from the cytoplasmic solution by an electrostatic attractive mechanism (Linsdell, 2005). This lysine residue is thought to reside within a relatively wide inner vestibule in the pore, on the cytoplasmic side of a narrow pore region that is the main determinant of selectivity between different anions (Linsdell, 2006). Recently we have identified other positively charged amino acids – arginine resiudes R303 and R352 – as contributing functionally important surface charges to the intracellular mouth of the pore (St. Aubin and Linsdell, 2006). Thus the positive charges contributed by these resiudes act to concentrate Cl^- ions close to the cytoplasmic mouth of the pore, ensuring a ready supply of Cl^- ions to enter the wide pore inner vestibule (St. Aubin and Linsdell, 2006).

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In the present study, we have investigated the mechanism of action of suramin, a large polyvalent organic anion that is known to be a potent inhibitor of CFTR Cl^- currents when applied to the intracellular side of the membrane (Bachmann et al., 1999). We show that suramin causes a biophysically distinct open channel block with a novel molecular mechanism that involves electrostatic interaction with R303. Based on our results, we present a model whereby the pore contains at least two distinct binding sites for blockers, and suggest that differences in the interaction between blocking anions and these two sites result in functional differences in the mechanism of block observed.

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MATERIALS AND METHODS

Experiments were carried out on baby hamster kidney (BHK) cells transiently transfected with wild-type or mutant forms of CFTR (Gong et al., 2002a). Macroscopic and single channel patch clamp recordings were made from inside-out membrane patches excised from these cells, as described in detail previously (Gong et al., 2002a; Gong and Linsdell, 2003; St. Aubin and Linsdell, 2006). After patch excision and recording of background currents, CFTR channels were activated by exposure to protein kinase A catalytic subunit (PKA) plus MgATP (1 mM) in the cytoplasmic solution. As in previous studies on these cells (Ge et al., 2004; St. Aubin and Linsdell, 2006), single channel currents were recorded after weak PKA stimulation (1-5 nM) whereas all macroscopic CFTR currents were recorded after maximal PKA stimulation (~20 nM) and subsequent treatment with sodium pyrophosphate (PPi; 2 mM) to "lock" channels in the open state. In all experiments the intracellular (bath) solution contained (mM): 150 NaCl, 2 $MgCl_2$, 10 *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES). While a few experiments (Fig. 3) used the same solution in the extracellular (pipette) solution, all other macroscopic and single channel experiments used a low Cl^- extracellular solution in which NaCl was replaced by Na gluconate. All experimental solutions were adjusted to pH 7.4 using NaOH. All chemicals were from Sigma-Aldrich (Oakville, ON, Canada) except PKA (Promega, Madison, WI) and DNDS (Molecular Probes, Eugene, OR). Channel blockers were prepared as described previously (Linsdell, 2005). Suramin was initially prepared as a high concentration aqueous stock solution and diluted in intracellular solution prior to addition to the experimental chamber. Structures of the six CFTR inhibitors used in this study are shown in Fig. 1.

Current traces were filtered at 100 Hz using an 8-pole Bessel filter, digitized at 250 Hz (for macroscopic currents) or 1 kHz (for single channel currents), and analyzed using pCLAMP

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software (Molecular Devices, Sunnyvale, CA). Single channel open times were measured using a 50% threshold detection method. Because single channel activity was recorded under conditions of very weak PKA stimulation (see above), patches contained a large number of CFTR channels with very low open probability. As a result, we have not analyzed channel burst duration or closed times, but instead focussed entirely on open time within a burst, which we assume to be relatively independent of the overall level of channel activity. Macroscopic current-voltage relationships were constructed using depolarizing voltage ramp protocols (Linsdell and Hanrahan, 1996a, 1998). Background (leak) currents recorded before addition of PKA have been subtracted digitally, leaving uncontaminated CFTR currents (Linsdell and Hanrahan, 1998; Gong and Linsdell, 2003). Given voltages have been corrected for liquid junction potentials calculated using pCLAMP software.

For macroscopic current inhibition by suramin, concentration-inhibition relationships were fitted by the equation:

$$\text{Fractional unblocked current} = 1 / (1 + ([\text{suramin}]/K_d)^{nH}) \quad [1]$$

where K_d is the apparent blocker dissociation constant and nH the slope factor or Hill coefficient. The effects of other blockers on wild type and mutant CFTR channels have been described previously (Linsdell, 2005), and for these relatively well-characterized blockers (glibenclamide, DNDS, lonidamine, NPPB, TLCS) relative effects on different channel variants were compared at a single concentration of blocker, as described previously (Linsdell, 2005). In these cases, the K_d was simply approximated using the equation:

$$K_d = [B] / ((1 / (I/I_0)) - 1) \quad [2]$$

where [B] is the concentration of blocker, I is the current amplitude in the presence of blocker, and I_0 the control, unblocked current amplitude.

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RESULTS

Inhibition of Wild-type CFTR by Intracellular Suramin

Suramin, a large organic molecule that bears a total of six negative charges (Fig. 1), has been shown to be a potent inhibitor of CFTR Cl^- channel currents when applied to the cytoplasmic face of the channel (Bachmann et al., 1999). However, little is known about its mechanism of action. Previously we have compared the effects of a number of different CFTR open channel blockers applied to the intracellular face of membrane patches excised from BHK cells (Linsdell, 2005). Fig. 2 shows the effects of intracellular suramin studied under the conditions previously described for these other blockers (Linsdell, 2005). Consistent with a previous report (Bachmann et al., 1999), suramin caused a potent inhibition of CFTR macroscopic Cl^- currents with a K_d of ~10 μM and no apparent voltage dependence. This draws an important distinction between suramin and well-characterized negatively charged CFTR open channel blockers, since these substances show voltage dependent inhibition by blocking currents far more potently at hyperpolarized than at depolarized potentials (see Introduction). Voltage dependence of inhibition is generally assumed to result from movement of the blocking substance into the channel pore and across part of the transmembrane electric field, and so is often used as evidence to support an open-channel blocking mechanism (Cai et al., 2004).

Negatively charged CFTR open channel blockers are also often sensitive to the extracellular (*trans*-) Cl^- concentration, whereby extracellular Cl^- ions antagonize their inhibitory effects, perhaps due to ion-ion interactions taking place within the pore (see Introduction). Again, this property has been used as evidence for an open channel block mechanism of action of some CFTR inhibitors (Linsdell and Gong, 2002; Cai et al., 2004). The effect of extracellular Cl^- ions on inhibition by intracellular suramin is shown in Fig. 3. The inhibitory effects of 10 μM

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suramin were practically identical whether the extracellular Cl^- concentration was 4 mM or 154 mM (Fig. 3B), again drawing an apparent distinction between suramin and well-defined CFTR open channel blockers.

At the single channel level, suramin caused brief interruptions in the open channel current without apparently reducing unitary current amplitude (Fig. 4A). The appearance of these short closed events led to a decrease in the mean channel open time in the presence of suramin (Fig. 4B), giving mean open time constants of 479 ± 66 ms (n = 3) for control and 78 ± 19 ms (n = 4) with 6 μM suramin ($P < 0.05$, two-tailed t-test). While we have not characterized the effects of suramin on single channel kinetics in great detail, we note that similar brief interruptions of the open channel current are observed with several CFTR open channel blockers (McCarty et al., 1993; Sheppard and Robinson, 1997; Cai et al., 1999; Zhang et al., 2000; Gong et al., 2002b), and in these cases it is assumed that each brief soujourn to the closed current level represents an individual blockage of the open pore (Cai et al., 2004). However, since we have not investigated in detail the gating of CFTR channels (see Materials and Methods), we cannot rule out that suramin also has inhibitory effects on channel opening.

Molecular Determinants of Suramin Inhibition

Structurally diverse, negatively charged organic molecules act as CFTR open channel blockers, apparently due in large part to electrostatic interactions with a positively charged lysine side chain, K95, located within the channel pore (Linsdell, 2005). Thus the point mutation K95Q greatly weakened the blocking effects of glibenclamide, DIDS, lonidamne, NPPB, and TLCS (Linsdell, 2005). In contrast, inhibition by intracellular suramin was unaffected by mutagenesis

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of this lysine residue (Fig. 5), suggesting that suramin does not share the same molecular mechanism of action as these other substances.

Recently, we showed that other positively charged amino acid residues, R303 and R352, appear to be located at the cytoplasmic mouth of the CFTR channel where they act to attract Cl^- ions into the pore by an electrostatic mechanism (St. Aubin and Linsdell, 2006). As shown in Fig. 5, a mutation that removed one of these positive charges, R303Q, significantly weakened the inhibitory effects of suramin. The relative effects of other amino acid substitutions at this position on the K_d for suramin inhibition are shown in Fig. 6. The greatest weakening of inhibition was observed in the side chain charge-reversing R303E mutant, whereas the charge-conserving R303K mutant did not significantly affect the K_d for suramin inhibition (Fig. 6). This apparent side chain charge-dependence of block suggests that the negatively charged suramin molecule makes an electrostatic interaction with this residue at the intracellular entrance to the pore. Unfortunately, since CFTR channels bearing mutations at R352 show low levels of expression in BHK cells (St. Aubin and Linsdell, 2006, and unpublished observations), we were unable to investigate the role of this residue in determining suramin sensitivity of the channel.

Interaction of R303 with Other Channel Blockers

Previously we showed that K95 contributed to CFTR open channel block by glibenclamide, but suggested that other amino acid residues in the pore might also influence glibenclamide binding in the pore inner vestibule (Linsdell, 2005). Since R303 is predicted to lie between K95 and the inner mouth of the pore (St. Aubin and Linsdell, 2006), we wondered if R303 might also play a role in glibenclamide block. Fig. 7 compares the blocking effects of intracellular glibenclamide in wild type CFTR with the charge-neutralizing mutants R303Q and

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K95Q. It can be seen that glibenclamide inhibition is weakened in R303Q, although the effects of this mutant are not as dramatic as those seen in K95Q. Furthermore, a double mutant in which both of these positively charged residues were mutated to neutral glutamines (K95Q/R303Q) showed similar glibenclamide sensitivity as K95Q alone (Fig. 7).

Lysine 95 also plays a strong role in open channel block by other substances, namely DNDS, lonidamine, NPPB and TLCS (Linsdell, 2005), leading us to suggest that these structurally diverse open channel blockers share a common molecular mechanism of action, binding close to K95 and plugging the open CFTR channel pore (Linsdell, 2005). As shown in Fig. 8, DNDS block was also weakened in R303Q; however, this mutation did not significantly affect block by lonidamine, NPPB or TLCS. The overall effects of the R303Q mutation on the affinity of block by the six different substances used in the present study are summarized in Fig. 9 and Table 1; the structures of these six substances are shown in Fig. 1.

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DISCUSSION

Like many other organic anions, suramin inhibits CFTR Cl^- currents when applied to the cytoplasmic side of the membrane (Bachmann et al., 1999; Fig. 2). It has been reported to be ineffective when applied to the outside of the cell (Bachmann et al., 1999). This sidedness is similar to that observed with many open channel blockers (Linsdell, 2006), but would also be predicted of allosteric inhibitors that target the intracellular gating machinery of CFTR. Indeed, several features of suramin inhibition are unlike those of well-characterized CFTR open channel blockers such as those described in Introduction. Thus suramin, in spite of its large net charge (-6), shows practically no voltage dependence (Figs. 2, 3) and appears insensitive to the extracellular Cl^- concentration (Fig. 3). This contrasts with many different open channel blockers, the inhibitory effects of which are strengthened by hyperpolarization of the membrane potential and weakened by extracellular Cl^- ions (McDonough et al., 1994; Linsdell and Hanrahan, 1996b; Sheppard and Robinson, 1997; Linsdell and Hanrahan, 1999; Gong et al., 2002b; Zhou et al., 2002; Gong et al., 2003). In fact, this voltage- and *trans*- Cl^--dependence of block can be used to discriminate between open channel blockers and allosteric inhibitors (Cai et al., 2004). Thus the biophysical properties of suramin block that we observe do not allow us to differentiate between an allosteric blocking mechanism or a functionally distinct form of open channel block.

Diverse CFTR open channel blockers including sulfonylureas, arylaminobenzoates, disulfonic stilbenes, indazoles, and conjugated bile salts (see Fig. 1) show a common molecular mechanism of action, interacting strongly with a positively charged lysine residue, K95, located in the wide pore inner vestibule (Linsdell, 2005). In contrast, removal of this positive charge by mutagenesis has no effect on suramin inhibition (Figs. 5, 6), suggesting that the large, polyvalent

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suramin molecule does not enter deeply enough into the pore from its cytoplasmic end to experience electrostatic interactions with K95. However, suramin inhibition is greatly weakened by mutagenesis of another positively charged residue, R303 (Figs. 5, 6), that is located at the cytoplasmic mouth of the pore (St. Aubin and Linsdell, 2006). We propose that suramin binds at the intracellular pore mouth, near R303, to inhibit Cl^- permeation. Furthermore, based on the apparent dependence of block on side chain charge at this position (Fig. 6), we suggest that the the positively charged R303 residue and the negatively charged suramin molecule interact in an electrostatic manner. We therefore propose that the pore inner vestibule has two blocker binding sites – a relatively deep site including K95, and a more superficial site involving R303 (Fig. 10).

Mutations that remove the positive charge at R303 lead to a reduction in Cl^- conductance due to a reduction in attractive surface charge effects (St. Aubin and Linsdell, 2006). Substances that bind to R303 – especially those that carry a large net negative charge – might be able to reproduce the effects of mutagenesis, by "screening" the important positive charge on the R303 side chain and so masking its attractive effect on Cl^- ions. However, several reasons suggest that such surface charge screening is not the mechanism of action of suramin. First, alteration of the charge at R303 by mutagenesis or chemical modification leads to dramatic changes in the shape of the current-voltage relationship, because the positive charge at this position exerts a much greater influence on Cl^- entering the pore from its cytoplasmic end than from the outside (St. Aubin and Linsdell, 2006). Screening of the surface charge would be expected to have much the same effect, which would therefore lead to apparently voltage dependent blocking effects – inhibition would be much stronger at hyperpolarized voltages (where currents are carried by Cl^- efflux) than at depolarized voltages (current carried by Cl^- influx). In contrast to this scenario, inhibition by intracellular suramin shows practically no voltage dependence (Figs. 2, 3).

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Secondly, since surface charges alter the rate of Cl^- permeation, which is very rapid, mutations at R303 affect the single channel conductance (St. Aubin and Linsdell, 2006), and screening the surface charge at this position would be expected to have the same effect. Again, this is clearly not the mechanism of action of suramin, which introduces brief interruptions in the single channel current without changing unitary Cl^- conductance (Fig. 4). Our results therefore suggest that, rather than screening the important surface charge at the cytoplasmic pore mouth, suramin binding in this region physically occludes the pore at this point and briefly interrupts the flow of Cl^- ions through the pore. This effect may reflect the large size of the suramin molecule (see Fig. 1), which may be able to occlude the pore sufficiently at its cytoplasmic mouth to prevent the passage of Cl^- ions (Fig. 10B).

The charge-neutralizing R303Q mutation greatly weakens suramin block, significantly weakens the blocking effects of glibenclamide and DNDS (but to a lesser extent than for suramin), and has no apparent effect on block by lonidamine, NPPB, or TLCS (Fig. 9, Table 1). Thus lonidamine, NPPB and TLCS apparently experience no strong interaction with R303 as they pass through the cytoplasmic mouth of the pore to their more deeply situated binding site (Fig. 10A). The reasons for the minor effect of the R303Q mutation on inhibition by glibenclamide and DNDS are less clear. Block by these substances is much more sensitive to removal of the positive charge at K95 (Fig. 7; Table 1; Linsdell, 2005), consistent with their primary mechanism of action being to block the pore at this deeper level (Fig. 10A). One possibility is that the positive charge of R303 acts to attract negatively charged glibenclamide and DNDS molecules into the pore inner vestibule, in much the same way that this important surface charge acts to attract Cl^- ions into the pore (St. Aubin and Linsdell, 2006). Removal of this surface charge in R303Q would then reduce the rate of glibenclamide and DNDS entry into

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the pore and to their primary binding site near K95. Alternatively, it may be that glibenclamide and DNDS also bind near R303, and that their binding at this superficial site also leads to a reduction in Cl^- permeation. Effectively this would mean that glibenclamide and DNDS have at least two binding sites at which they can block the open channel. Indeed, it has been proposed that glibenclamide inhibition of CFTR is complex and may involve multiple blocking mechanisms, perhaps with distinct molecular bases (Zhang et al., 2004). However, the effects of mutagenesis at K95 and R303 on glibenclamide inhibition do not appear to be additive (Fig. 7), suggesting that glibenclamide interactions with these two sites may not be completely functionally independent. A further possibility is that both the glibenclamide and DNDS molecules might be able to interact simultaneously with K95 and R303. It has been suggested that different parts of the glibenclamide molecule might interact with different parts of the pore (Cai et al., 1999; Zhang et al., 2004; Linsdell 2005). However, since glibenclamide carries only a single negative charge it is difficult to envisage how it could interact electrostatically with two positive charges located in different parts of the pore at the same time. The DNDS molecule, on the other hand, has two negative charges that are located on different parts of the molecule, making it possible that it could show a bidentate interaction with K95 and R303. Whatever the mechanism, the far greater effects of the K95Q mutation on block by glibenclamide and DNDS relative to R303Q (Table 1) are consistent with the most important interaction underlying open channel block by these two molecules being with K95.

The model illustrated in Fig. 10 suggests that the pore contains at least two sites at which organic anion binding can produce current inhibition by an open channel block mechanism. Substances that are able to penetrate deeply into the pore interact with the positively charged side chain of K95 to produce an open channel block (Fig. 10A) that is sensitive both to the membrane

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potential (suggesting that this amino acid residue is situated within the transmembrane electric field) and the *trans*- (extracellular) Cl^- concentration (perhaps because that this residue is located close enough to Cl^- ions present in more extracellular pore regions – such as the narrow pore region or putative "selectivity filter" (Linsdell, 2006) – that blocking anions bound here can sense these Cl^- ions electrostatically). In contrast, substances that cannot penetrate deeply enough into the pore to interact with K95 may still cause open channel block if they are able to occlude the pore at the level of R303 (Fig. 10B). Such a blocking reaction appears to be insensitive both to the membrane potential (perhaps suggesting that R303, at the cytoplasmic pore mouth, is located outside of the transmembrane electric field) and to *trans*-Cl^- ions (perhaps suggesting that Cl^- ions bound within the pore are too far away to influence the blocking reaction). The most likely factor determining whether or not an organic anion is able to pass from the superficial site near R303 to the deep site near K95 would seem to be its size, suggesting that the pore inner vestibule narrows between the locations of these two positively charged amino acids (Fig. 10).

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1 **Structures of the six CFTR inhibitors used.**

Figure 2 **Inhibition of CFTR Cl^- currents by intracellular suramin.** *A*, Example leak-subtracted current-voltage relationship recorded from an inside-out membrane patch following maximal current stimulation with PKA and PPi. Current was recorded before (control) and after (+ suramin) addition of 10 μM suramin to the intracellular solution. *B*, Mean fraction of control current remaining (I/I_0) after the addition of different concentrations of suramin, at membrane potentials of –100 mV (●) and 0 mV (❍). Mean of data from 5 patches. The fitted lines are to Eq. [1] for the data at –100 mV (solid line), giving a K_d of 9.5 μM and nH of 1.30, and at 0 mV (dashed line), giving a K_d of 8.9 μM and nH of 1.27. *C*, Suramin inhibition shows no apparent voltage dependence. K_d values were estimated at each voltage as described in *B*.

Figure 3 **Suramin inhibition is independent of extracellular Cl^-.** *A*, Example leak-subtracted current-voltage relationship recorded using symmetrical 154 mM Cl^--containing solution, before (control) and after (+ suramin) addition of 10 μM suramin to the intracellular solution. *B*, Mean fraction of control current remaining (I/I_0) after addition of 10 μM suramin, at extracellular Cl^- concentrations of 4 mM (●) and 154 mM (❍), plotted as a function of membrane potential. Mean of data from 4-5 patches.

Figure 4 **Effects of intracellular suramin on CFTR single channel currents.** *A*, Example single channel currents recorded from inside-out patches at a membrane potential of -30 mV, in the absence (control) or presence of 6 μM suramin (+ suramin). In each case the closed state of the channel is indicated by the line on the far left. *B*, Open time histograms obtained from long single channel recordings such as those shown in *A*. Both have been fit with a single exponential decay function with time constants of 439 ms (control) and 73 ms (+ suramin).

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Figure 5 **Suramin inhibition of CFTR channel mutants.** *A*, Example leak-subtracted current-voltage relationships for K95Q and R303Q-CFTR, recorded before (control) and after (+ suramin) addition of 10 μM suramin to the intracellular solution. *B*, Mean fraction of control current remaining (I/I_0) after the addition of different concentrations of suramin, at a membrane potentials of –100 mV, in K95Q (●) and R303Q (❍). Mean of data from 5-6 patches, fitted by Eq. [1] (solid lines), giving K_ds of 11.6 μM for K95Q and 48.2 μM for R303Q. For comparison, the fit to the data for wild type under these conditions (see Fig. 2B) is shown as a dotted line.

Figure 6 **Effect of channel mutants on the affinity of suramin inhibition.** Mean K_d was estimated at –100 mV as described in Figs. 2B and 5B for different channel variants and ionic conditions. Mean of data from 4-6 patches. * indicates a significant difference from wild type (at low extracellular Cl^-), $P < 0.01$ (two-tailed *t*-test).

Figure 7 **Relative roles of pore-forming positively charged amino acids on channel inhibition by intracellular glibenclamide.** *A*, Example leak-subtracted current-voltage relationships for wild type, R303Q, K95Q and the K95Q/R303Q double mutant, recorded before (control) and after (+ glibenclamide) addition of 30 μM glibenclamide to the intracellular solution. *B*, Mean fraction of control current remaining (I/I_0) after the addition of this concentration of glibenclamide. In each panel, data for the channel mutant in question (●) is shown along with wild type (❍) for comparison. *C*, Mean K_d for glibenclamide inhibition at –100 mV, estimated according to Eq. [2]. * indicates a significant difference from wild type, $P < 0.0001$ (two-tailed *t*-test). Mean of data from 11 patches for wild type and 4-5 patches for different mutants in *B* and *C*.

Figure 8 **Inhibition of R303Q-CFTR by open channel blockers.** *A*, Example leak-subtracted current-voltage relationships for wild type and R303Q-CFTR, recorded before

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(control) and after addition of the named blocker to the intracellular solution: DNDS (100 μM), lonidamine (100 μM), NPPB (50 μM) or TLCS (50 μM). *B*, Mean fraction of control current remaining (I/I_0) after the addition of these concentrations of blockers in wild type (❍) and R303Q (●). Mean of data from 3-4 patches.

Figure 9 Role of R303 in determining the potency of CFTR channel inhibitors. Mean K_d values estimated at –100 mV for each of the six CFTR inhibitors used in the present study under identical ionic conditions, for wild type (open bars) and R303Q (filled bars). * indicates a significant difference from wild type, $P < 0.01$ (two-tailed *t*-test). Mean of data from 3-11 patches.

Figure 10 Proposed arrangement of blocker binding sites in the CFTR pore. We propose that the pore inner vestibule contains at least two distinct blocker binding sites. *A*, The deeper site, which includes K95, is visited by smaller organic open channel blocker molecules. *B*, Occupancy of the more superficial site, which includes R303, by large organic anions may also lead to open channel block. The relative positions of K95 and R303 within the pore also lead to differences in the voltage-dependence and extracellular Cl^- concentration-dependence of open channel blocking reactions at the two sites.

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TABLES

Table 1 Relative effect of removal of positive charges in the pore on the inhibitory effects of different channel blockers

Blocker	K95Q	R303Q
Suramin	0	++
Glibenclamide	++	+
DNDS	++	+
Lonidamine	++	0
NPPB	++	0
TLCS	++	0

The effect of point mutations in the pore on the apparent affinity of block by different substances (the structures of which are given in Fig. 1) were grouped according to their effect on K_d : 0, <2 fold change in K_d; +, 2-8 fold increase in K_d; ++, >8 fold increase in K_d. Data for block of K95Q by DNDS, lonidamine, NPPB and TLCS taken from Linsdell (2005).

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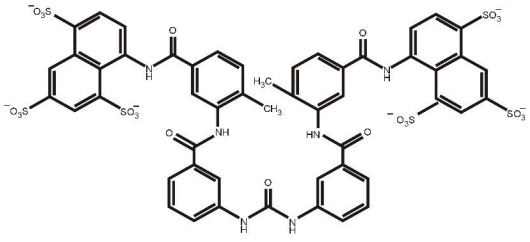
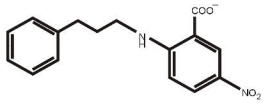
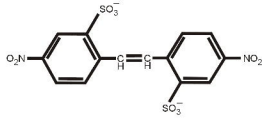


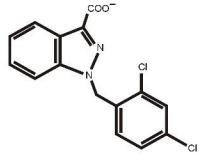
Suramin



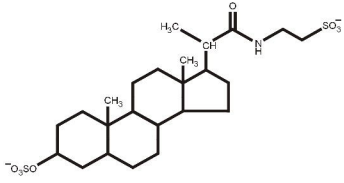
NPPB



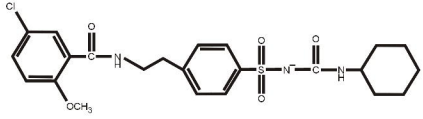
DNDS



Lonidamine



TLCS



Glibenclamide

Figure 1

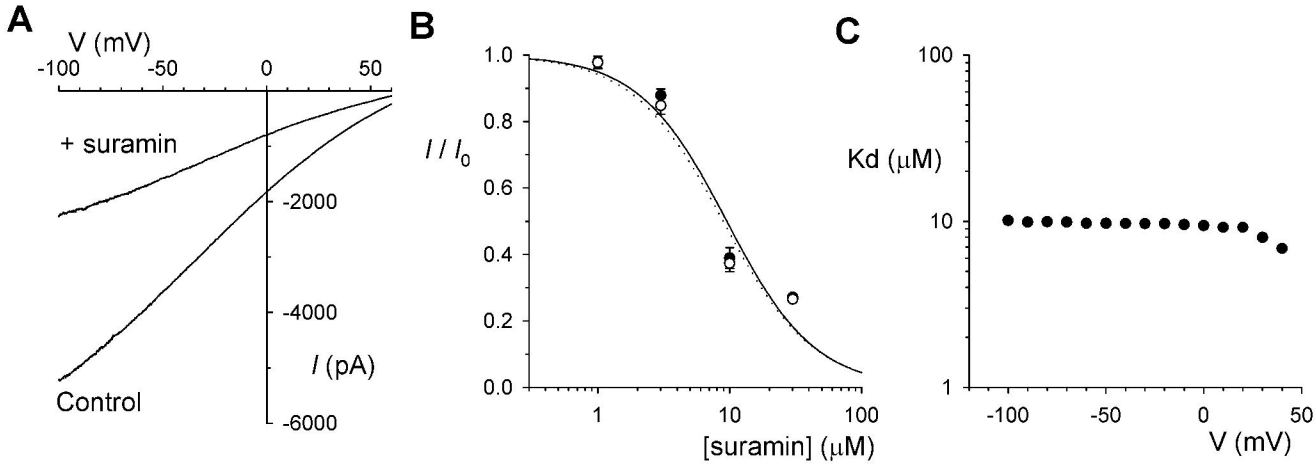

A
V (mV)
-100
-50
0
50
+ suramin
-2000
-4000
I (pA)
Control
-6000
B
1.0
0.8
0.6
0.4
0.2
0.0
I / I0
1
10
100
[suramin] (μM)
C
100
Kd (μM)
10
1
-100
-50
0
50
V (mV)

Figure 2

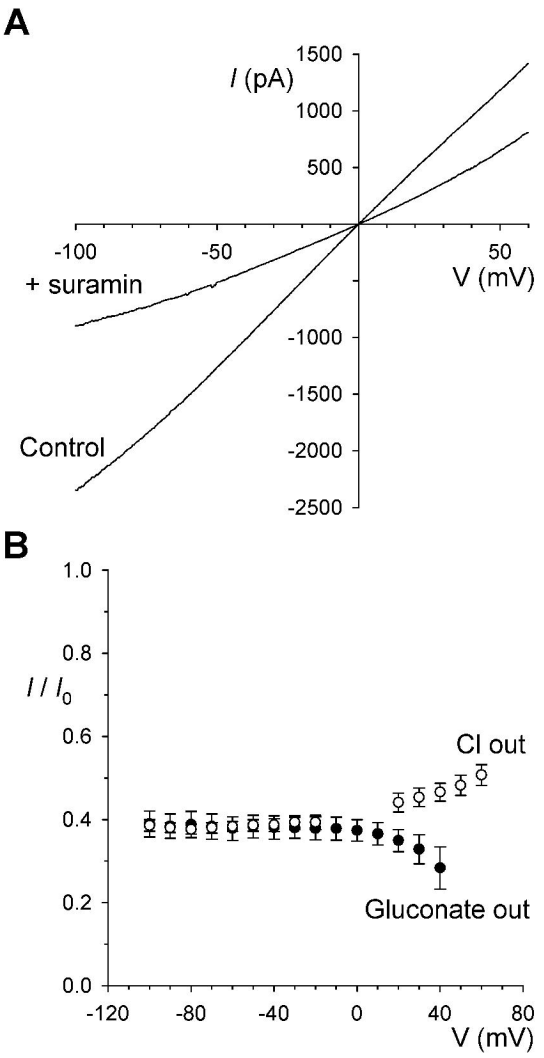

A
I (pA)
1500
1000
500
-100
-50
50
V (mV)
+ suramin
Control
-1000
-1500
-2000
-2500
B
1.0
0.8
I / I0
0.6
0.4
0.2
0.0
Cl out
Gluconate out
-120
-80
-40
0
40
80
V (mV)

Figure 3

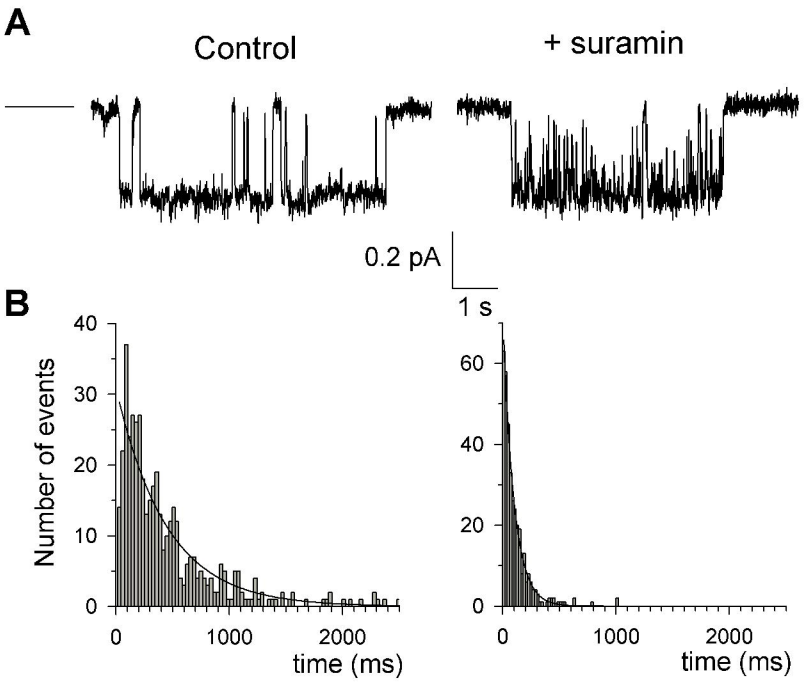

A
Control
+ suramin
0.2 pA
1 s
B
Number of events
40
30
20
10
0
0
1000
2000
time (ms)
60
40
20
0
0
1000
2000
time (ms)

Figure 4

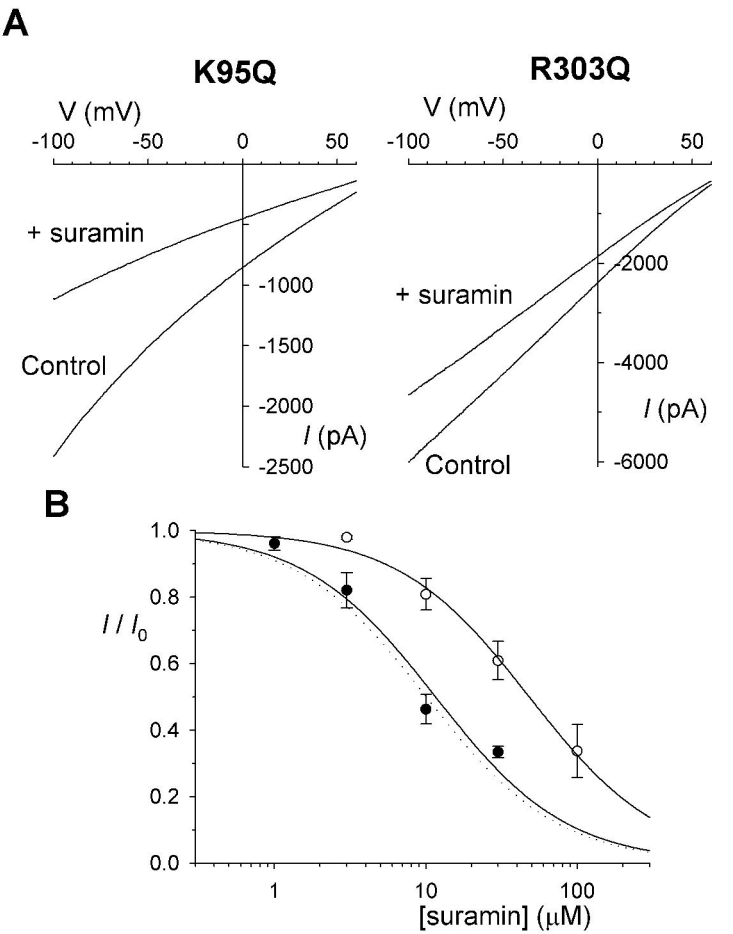

A
K95Q
R303Q
V (mV)
-100
-50
0
50
+ suramin
Control
-1000
-1500
-2000
-2500
I (pA)
-2000
-4000
-6000
B
I / I0
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
[suramin] (μM)

Figure 5

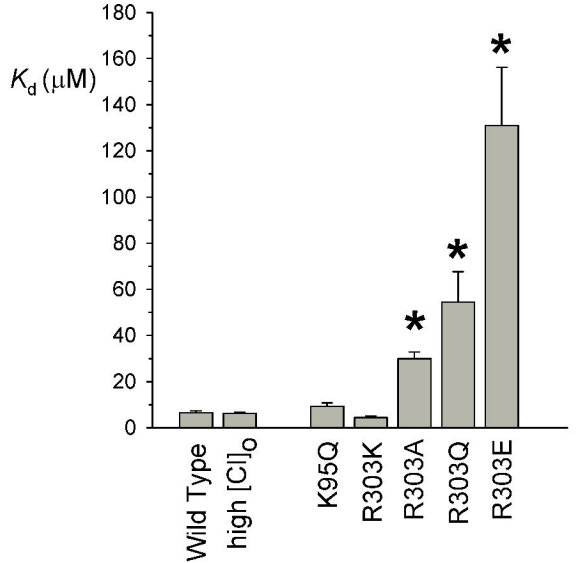

K_d (μM)
180
160
140
120
100
80
60
40
20
0
*
*
*
Wild Type
high $[Cl]_o$
K95Q
R303K
R303A
R303Q
R303E

Figure 6

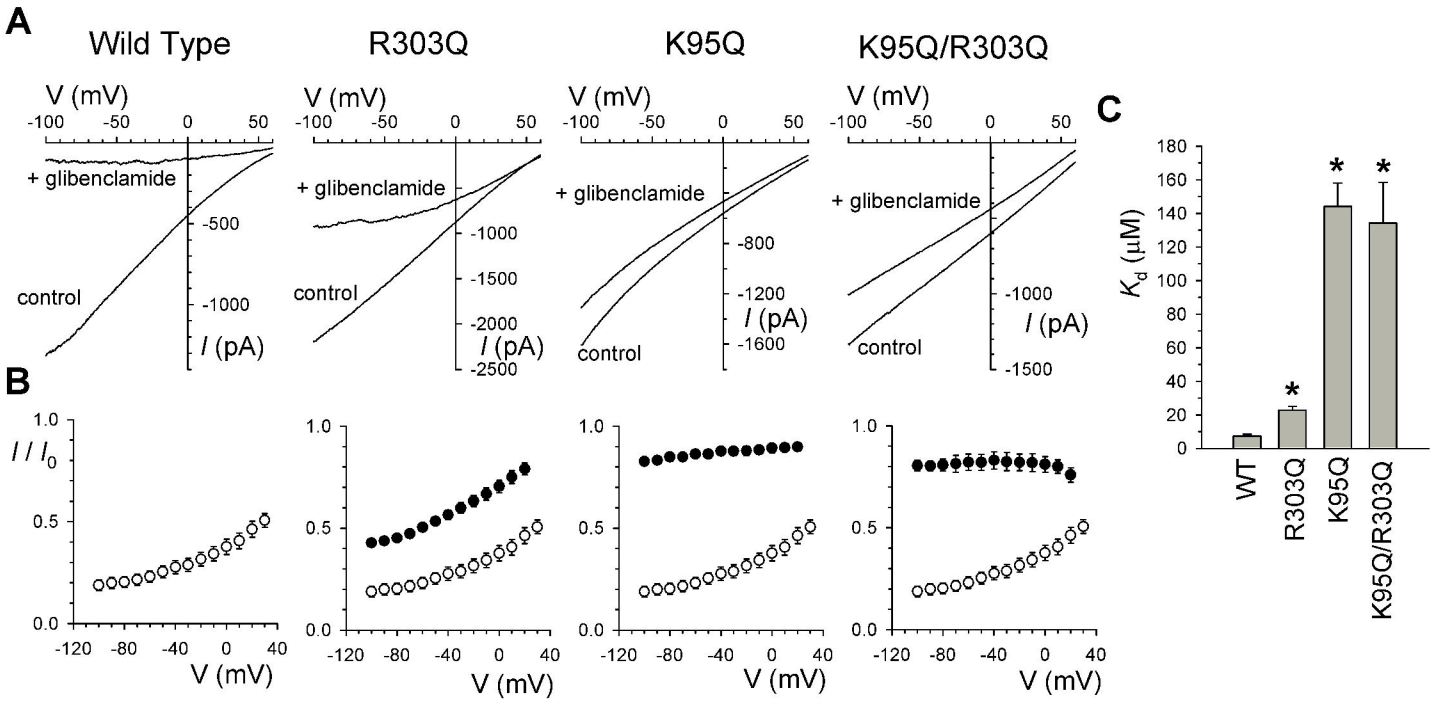


Figure 7

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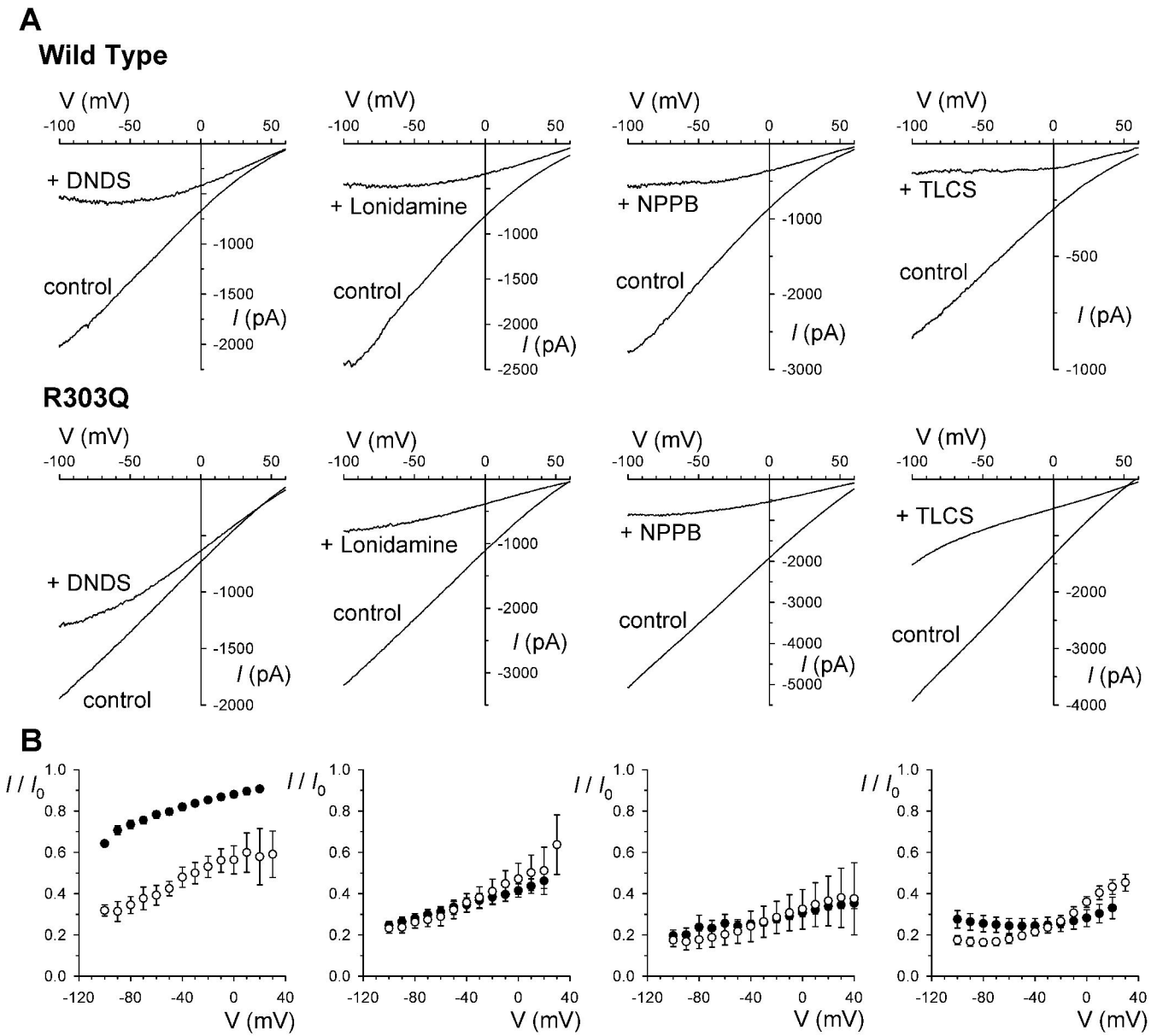
A
Wild Type
V (mV)
-100 -50 0 50
+ DNDS
control
-1000
-1500
-2000
I (pA)
+ Lonidamine
-2500
+ NPPB
-3000
+ TLCS
-500
-1000
R303Q
-4000
-5000
B
I / I0
1.0
0.8
0.6
0.4
0.2
0.0
-120 -80 -40 0 40
V (mV)

Figure 8

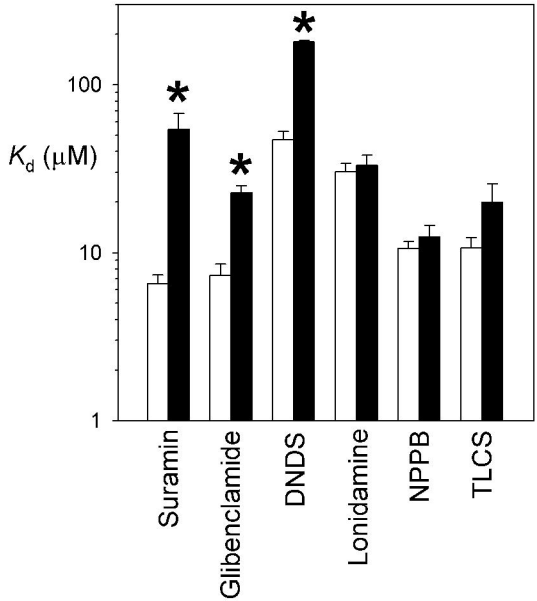
100
10
1
K_d (μM)
*
*
*
Suramin
Glibenclamide
DNDS
Lonidamine
NPPB
TLCS

Figure 9

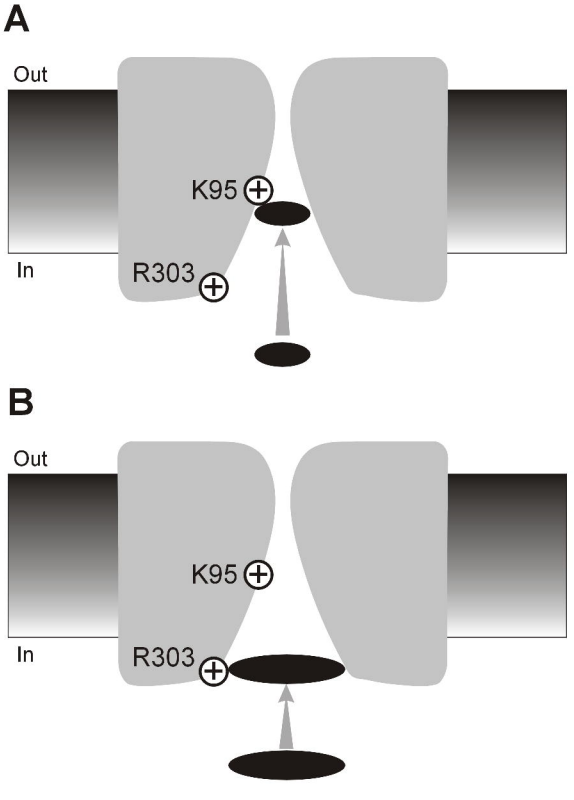

Figure 10