Functional organization of cytoplasmic portals controlling access to the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel pore

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl\(^-\) channel that apparently has evolved from an ancestral active transporter. Key to the CFTR’s switch from pump to channel function may have been the appearance of one or more “lateral portals.” Such portals connect the cytoplasm to the transmembrane channel pore, allowing a continuous pathway for the electrodiffusional movement of Cl\(^-\) ions. However, these portals remain the least well-characterized part of the Cl\(^-\) transport pathway; even the number of functional portals is uncertain, and if multiple portals do exist, their relative functional contributions are unknown. Here, we used patch-clamp recording to identify the contributions of positively charged amino acid side chains located in CFTR’s cytoplasmic transmembrane extensions to portal function. Mutagenesis-mediated neutralization of several charged side chains reduced single-channel Cl\(^-\) conductance. However, these same mutations differentially affected channel blockade by cytoplasmic suramin and Pt(NO\(_2\))\(_4\)\(^{2-}\) anions. We considered and tested several models by which the contribution of these positively charged side chains to one or more independent or non-independent portals to the pore could affect Cl\(^-\) conductance and interactions with blockers. Overall, our results suggest the existence of a single portal that is lined by several positively charged side chains that interact electrostatically with both Cl\(^-\) and blocking anions. We further propose that mutations at other sites indirectly alter the function of this single portal. Comparison of our functional results with recent structural information on CFTR completes our picture of the overall molecular architecture of the Cl\(^-\) permeation pathway.

Cystic fibrosis is caused by loss-of-function mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial cell Cl\(^-\) channel. CFTR is a member of the ATP-binding cassette family of membrane transport proteins, most members of which function as ATP-dependent pumps (1, 2). It is presumed that some evolutionary change has allowed CFTR to function not as a pump but as an ion channel (3–5). Specifically, as an ion channel, CFTR must exhibit a continuous aqueous pathway that allows free electrodiffusional movement of Cl\(^-\) and other small anions between the cytoplasm and the extracellular solution in the channel open state. Such a continuous open pathway is incompatible with the function of a pump.

As with other ATP-binding cassette proteins, CFTR is made up of two membrane-spanning domains each comprising six transmembrane (TM) segments and two cytoplasmic nucleotide binding domains (NBDs). The TMs form long extensions into the cytoplasm (TMEs) to contact the NBDs. Recently the molecular structure of CFTR was observed using cryo-electron microscopy (cryo-EM), first in an inactive, closed state with separated NBDs (Fig. 1A) (6, 7) and subsequently in a “near-open” state with dimerized NBDs (Fig. 1D) (8). Both of these structures show a continuous transmembrane channel pore that is wide at its cytoplasmic end and apparently closed at its extracellular end (7, 8). These structural data are in broad agreement with longstanding functional models of the channel pore and with a large body of structure–function work identifying the molecular architecture of the pore (9). Briefly, functional models suggest that the transmembrane part of the pore is made up of a wide inner vestibule that is accessible from the cytoplasm both in open and in closed channels, a narrow region that forms the anion selectivity filter, and a shallower outer vestibule accessible from the extracellular solution (9).

Comparison of the structures of CFTR in “closed” (Fig. 1, A and B) and “open” (Fig. 1, D and E) states is consistent with NBD dimerization resulting in channel opening and drawing together of the inner ends of the TMEs. The large-scale rearrangement of the TMEs implied by Fig. 1 (A and D) suggests that cytoplasmic substances might be able to access the pore-forming TMs via different routes in closed channels and in open channels. It is known that large cytoplasmic substances can access the inner vestibule of the pore lined by the TMs not only in the open state, but also when the channel is closed (10–14). Although there appears to be a central pathway between the TMEs that allows free access to the TM pore in the closed state (Fig. 1, A–C), tight closure of the inner ends of the TMEs appears to close off this pathway in the open state (Fig. 1, D and E). Instead, access to the TM pore is thought to occur via one or more lateral portals or tunnels formed between individual

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2 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; cryo-EM, cryo-electron microscopy; NBD, nucleotide binding domain; PKA, protein kinase A; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; TM, transmembrane helix; TME, transmembrane helical extension.

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TMEs (8, 9, 15–18) (Fig. 1F). The existence of up to four such portals within the TMEs was originally postulated based on atomic homology modeling (15, 16). Based on the effect of mutations and chemical modifications of different amino acids on Cl− conductance, we suggested that one or two portals may be involved in Cl− access to the pore: a functionally major portal formed between TMEs 4 and 6 and perhaps a functionally lesser portal on the opposite side of the protein, between TMEs 10 and 12 (17). In particular, we suggested that positively charged amino acid side chains located in several different TMEs (Fig. 1, B and E) attracted cytoplasmic Cl− ions to the pore electrostatically (17). Although the cryo-EM structure of Fig. 1D appears to confirm the presence of a portal between TMEs 4 and 6, only a small gap of unknown functional importance was identified between TMEs 10 and 12 (8). Furthermore, positive electrostatic potential around this single large portal supports the idea that it is involved in electrostatic attraction of cytoplasmic Cl− ions (8).

In addition to their functional importance in Cl− conduction (17, 18), the portals formed by the TMEs may also be the site of action of some CFTR inhibitors. In particular, it was shown that Arg303 in TME5 interacts electrostatically with the large channel blocking anion suramin (19). Furthermore, it has been proposed that the appearance of the portals may have been a key step in the evolution of CFTR from a pump into an ion channel (5). Despite their functional importance and mechanistic significance, the portals remain the least well-characterized part of the CFTR permeation pathway. As described above, even the number of portals utilized by Cl− ions to access the pore remains open to question, and if more than one portal exists, their relative functional contributions remain unknown. In this work we have studied the effect of mutating positively charged residues potentially contributing to portal function (Fig. 1, B and E) on both Cl− conductance and block by cytoplasmic suramin and Pt(NO2)42− ions. Our results suggest that CFTR possesses one dominant lateral portal with a cytoplasmic entrance between TMEs 4 and 6 and that a number of positively charged side chains surrounding this portal act to attract cytoplasmic anions to the pore.

Results

Role of fixed positive charges in chloride conductance

Previously we showed that Arg303 in TME5 plays an apparently electrostatic role in interacting with a very large polyanionic blocker of CFTR, suramin (19). The blocking effects of intracellular suramin are compared for wildtype and charge-neutralizing mutant forms of CFTR in Fig. 3. Not only R303Q as shown previously (19) but also K190Q, R248Q, and K370Q significantly weakened the blocking effects of suramin, consistent with these positively charged side chains also normally interacting with negatively charged suramin molecules. In contrast, R1048Q had no significant effect on suramin inhibition, whereas two other mutations, K978Q and K1041Q, unexpectedly led to an apparent strengthening of channel current inhibition by intracellular suramin (Fig. 3). These results appear inconsistent with simple models of the cytoplasmic entrance to the pore (such as those shown in Fig. 1, C and F) in which all of these positively charged side chains act in a similar way to attract negatively charged Cl− and other anions (such as suramin) into the permeation pathway.

The effects of the K190Q, R248Q, R303Q, and K370Q mutations on Cl− conductance (Fig. 2) and suramin block (Fig. 3) are consistent with these positive charges attracting both Cl− and suramin to the pore. In contrast, the effects of K978Q, K1041Q, and R1048Q (Figs. 2 and 3) appear inconsistent with such a role for these residues. It is striking that Lys190, Arg248, Arg303, and Lys370 were all previously suggested to line a primary cytoplasmic portal to the pore (17) (Fig. 1E), whereas Lys1041 and Arg1048, as well as perhaps Lys978, are expected to lie closer to the opposite side of the channel (Fig. 1E) and potentially contribute to a secondary portal (17). The distinct effect of mutations at these two groups of sites led us to consider a model in which a differential contribution of these positively charged residues to the function of two separate portals could explain the similar effects of neutralizing mutations on Cl− conductance (Fig. 2) but the seemingly opposite effects of different mutations on suramin block (Fig. 3).

One model by which two portals could potentially explain these results is shown in cartoon form in Fig. 4A. In this model, both Cl− and suramin can enter the cytoplasmic part of the pore via a primary portal, whereas only Cl− can enter via a secondary portal. Although mutations affecting the function of the primary portal will then impair both Cl− conductance and suramin block, mutations affecting the secondary portal may reduce Cl− conductance but perhaps strengthen suramin block (for example, by reducing the relative contribution of a suramin-insensitive Cl− entrance pathway to the pore). This model would predict that the fraction of Cl− current that passes through the secondary portal should be resistant even to very high concentrations of suramin, potentially allowing the relative contributions of the two portals to Cl− conductance to be estimated from the suramin-insensitive fraction of the overall current. Furthermore, the suramin-insensitive component of the current might be increased in channel mutants that impaired Cl− entry through the main
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Figure 1. Structural and functional models of the cytoplasmic entrance to the CFTR channel pore. A, overall structure of human CFTR in the channel closed state (7). In this state, the TMs and TMEs are arranged in an “inward facing” conformation, such that the transmembrane permeation pathway is open to the cytoplasm and closed at its extracellular end. Those positively charged amino acid side chains that have been proposed, based on functional data, to attract cytoplasmic Cl⁻ ions to the permeation pathway are shown as space-filling models. These positive charges contribute to the overall positive electrostatic potential of this part of the protein (8). B, close-up view of the isolated TMEs (derived from A), showing the location of these positively charged side chains as labeled in the same colors. C, the wide-open nature of the TMEs suggests that Cl⁻ and other substances could be “funneled” toward the transmembrane pore in this closed state. D, overall structure of zebrafish CFTR in a near-open state (8). In this state, the TMEs are much more closely associated, and there is no “central” pathway from the cytoplasm to the pore (such as apparently exists in A). Positively charged amino acid side chains corresponding to those in A are shown using the same color scheme. E, close-up view of the isolated TMEs (derived from D). Note that the numbering of these positively charged amino acids is slightly different in zebrafish CFTR. F, tight closure of the inner ends of the TMEs led us to suggest that cytoplasmic Cl⁻ accesses the open channel pore by at least one, and possibly two, cytoplasmic portals that are lined by positively charged amino acid side chains that play an important role in electrostatic attraction of Cl⁻ to the permeation pathway (17).
and K370E showed a further decrease in suramin sensitivity compared with R303Q and K370Q (Fig. 6, B and D). In contrast, the suramin sensitivity of K1041E was similar to or slightly less than that of K1041Q (Fig. 6D). Again the most striking result was observed with K978E; whereas K978Q significantly strengthened suramin block compared with wildtype (Figs. 3C and 6D), suramin block of K978E was significantly weaker than for wildtype (p < 0.005, n = 5) (Fig. 6, B and D). Although these results further support an electrostatic role for Arg303 and Lys370 in interactions with anions, they do not support a purely electrostatic interaction between Lys978 and Lys1041 and either Cl− (Fig. 6C) or suramin (Fig. 6D).

To investigate the independence of effects of mutations at different sites, we also combined charge-neutralizing mutations predicted to be near the periphery (K370Q) and core (R303Q) of the primary portal and within the secondary portal (K1041Q). Overall, the double mutant channels R303Q/K370Q, R303Q/K1041Q, and K370Q/K1041Q had effects on CI− conductance (Fig. 6E) and suramin sensitivity (Fig. 6F) that appeared broadly additive of the effects of the individual mutations.

Role of fixed positive charges in Pt(NO2)4^2− block

In addition to determining the apparent affinity of suramin block (19), Arg303 has been proposed to play a role in channel block by smaller, divalent Pt(NO2)4^2− (22), even though these Pt(NO2)4^2− ions are able to penetrate much further into the pore than suramin and interact with sites within the TMs (22, 28). To test whether portal mutations would also have differential effects on Pt(NO2)4^2− block, we investigated suramin-resistant K370Q and suramin-sensitive K1041Q. As described previously for R303Q (22), K370Q led to a significant weakening of
block by intracellular Pt(NO$_2$)$_4$$^2$- ($p < 0.0005$; Fig. 7), consistent with Pt(NO$_2$)$_4$$^2$- entering the pore via the primary portal. In contrast, K1041Q showed significantly strengthened block by Pt(NO$_2$)$_4$$^2$- ($p < 0.005$; Fig. 7).

Discussion

Structural (7, 8), functional (17, 18), and molecular modeling studies (15, 16) concur that cytoplasmic Cl$^-$ ions use lateral portals, rather than a directly central route, to enter the CFTR channel protein and so begin their journey across the membrane. These portals may be a key factor in CFTR functioning as an ion channel rather than a pump (5). However, how these portals are arranged—and even how many portals exist and what their relative functional contributions to Cl$^-$ transport might be—remains to be defined. Previously we speculated, based on functional investigation, that two portals might exist (17). A functionally dominant portal was proposed between TMEs 4 and 6, and lined by Lys$^{190}$ (TME3), Arg$^{248}$ (TME4), Arg$^{303}$ (TME5), and Lys$^{370}$ (TME6) (17). We also speculated that a functionally lesser portal might be located on the opposite side of the protein, between TMEs 10 and 12 and possibly involving Lys$^{1041}$ and Arg$^{1048}$ in TME10 (17). This tentative model was based on the finding that mutation and chemical modification of amino acid side chains in different TMEs had qualitatively similar (although quantitatively different) effects on single channel Cl$^-$ current amplitudes (17). A possible contribution of positively charged amino acid side chains to distinct portals coming from opposite sides of the TMEs also appeared consistent with atomic homology models of that time (15, 17). However, we will argue that our present results investigating the effects of mutations of these same, functionally important positively charged side chains on both Cl$^-$ conductance and interactions with blockers are more consistent with the existence of a single portal from the cyto-
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Figure 4. Effect of high concentrations of suramin. A, hypothetical model (derived from Fig. 1F) in which both Cl\(^-\) (green) and suramin (red) could access the open pore via one “primary” portal (from the left as shown), whereas a “secondary” portal (from the right) allows access by Cl\(^-\) but not suramin (for example, because of restricted dimensions) (see “Results”). B, example macroscopic current–voltage relationships for the weakly suramin-sensitive mutant K370Q (left panel) and the strongly suramin-sensitive mutant K1041Q (right panel) in inside-out patches. In both cases, current was recorded before (control, black) and after (red) addition of 1 μm suramin to the intracellular solution. C, mean fraction of control current remaining after addition of different concentrations of suramin to the intracellular solution for the different channel variants indicated. The mean data have been fitted as described under “Experimental Procedures” (Equation 2) with the following parameters: wildtype \(K_d = 11.6 \mu M\), nh 0.87, \(rf = 0.047\); R303Q Kd 32.3 \(\mu M\), nh 0.93, \(rf = 0.06\); K370Q Kd 39.5 \(\mu M\), nh 1.10, \(rf = 0.034\); and K1041Q Kd 2.0 \(\mu M\), nh 1.19, \(rf = 0.060\). The asterisk indicates concentrations at which mutations showed a significant difference from wildtype (p < 0.05). At high concentrations (1–10 μm), none of the mutants studied showed a significant difference from wildtype. The means of data from 4–10 patches are shown.

plasm to the pore, with its mouth located between TMEs 4 and 6. This model appears consistent with the reported cryo-EM structure of the TMEs in the near-open state (Fig. 8A), which shows a large portal between TMEs 4 and 6 but not between TMEs 10 and 12 (8).

The cytoplasmic portal(s) appear to be the site of action of the large cytoplasmic channel blocker suramin (19) (Figs. 3–6). Investigation of the concentration dependence of suramin block (Fig. 4C) is consistent with this large anionic molecule occluding the entire Cl\(^-\) permeation pathway, most likely by interacting with a single binding site. However, the finding that charge-neutralizing mutations can either weaken or strengthen block by suramin (Figs. 3–6), as well as Pt(NO\(_2\))\(_4\) (Fig. 7), appears inconsistent with the simplest models in which all positively charged amino acid side chains studied electrostatically funnel Cl\(^-\) and blocking anions to the pore (Fig. 1, C and D).

All results concerning residues previously suggested to contribute to the primary cytoplasmic portal, namely Lys\(_{978}\), Arg\(_{248}\), Arg\(_{303}\), and Lys\(_{370}\) (17), do appear consistent with an electrostatic effect on the attraction of Cl\(^-\) (Figs. 2 and 6), suramin (Figs. 3 and 6), and Pt(NO\(_2\))\(_4\) (Fig. 7) (22) to the pore. These four positively charged residues may therefore line a \textit{bona fide} entry pathway into the pore from the cytoplasm, not only for Cl\(^-\) but also blocking anions such as suramin and Pt(NO\(_2\))\(_4\) (Fig. 8C). This is in fact consistent with the reported structure of the TMEs in the near-open state (Fig. 8A), which also shows a positive electrostatic potential surrounding the portal between TMEs 4 and 6 (8).

Although neutralization of other nearby positively charged residues (Lys\(_{978}\), Lys\(_{1041}\), and Arg\(_{1048}\)) also reduces Cl\(^-\) conductance (Fig. 2), other results argue against an electrostatic role of these residues in attracting anions to the pore. First, mutations at these sites either have no effect (Fig. 3) or actually strengthen block by suramin and Pt(NO\(_2\))\(_4\) (Figs. 3–7). Second, the effects of mutations at Lys\(_{978}\) do not appear to be related to amino acid side chain charge (Fig. 6, C and D) as would be expected of any kind of electrostatic effect. Mutations at Lys\(_{1041}\) also have effects that are only weakly related to side chain charge at best (Fig. 6, C and D), especially when compared with the strongly charge-dependent effects of mutations at Arg\(_{303}\) and Lys\(_{370}\) (Fig. 6, C and D). In fact, deposition of a negative charge at position Lys\(_{1041}\) or Arg\(_{1048}\) (by cysteine mutagenesis followed by modification with a negatively charged cysteine-reactive reagent) was previously shown to have only a small effect on Cl\(^-\) conductance when compared with Lys\(_{190}\), Arg\(_{248}\), Arg\(_{303}\), or Lys\(_{370}\) (17), again suggesting that these groups of positively charged residues had differential effects on pore function. Thus Lys\(_{978}\), Lys\(_{1041}\), and Arg\(_{1048}\) do not appear to influence channel properties by the same mechanism as Lys\(_{190}\), Arg\(_{248}\), Arg\(_{303}\), and Lys\(_{370}\).

We considered different models in which these amino acids might contribute to a secondary portal and thereby indirectly affect suramin binding (Figs. 4A and 5A). Several of our findings argue against the existence of such a secondary portal. As described above, mutation of these positively charged residues has effects on anion interactions with the channel that are either weakly charge-dependent or charge-independent. Furthermore, there does not appear to be a substantial suramin-insensitive component to the current (Fig. 4C) that might be expected for two independent entrances to the pore (Fig. 4A). In addition, suramin block of K1041Q remains strong even under low [Cl\(^-\)] conditions that minimize competition between suramin and permeant anions (Fig. 5G), arguing against the existence of two non-independent pore entrances (Fig. 5A). Finally, whereas mutations at Lys\(_{978}\), Lys\(_{1041}\), and Arg\(_{1048}\) clearly affect both Cl\(^-\) conductance (Figs. 2, 6) and suramin block (Figs. 3 and 6), there is no obvious pattern linking the effects of different mutations on these two aspects of pore function. Because we are unable to find compelling evidence that Lys\(_{978}\), Lys\(_{1041}\), and Arg\(_{1048}\) interact directly with suramin (or, in the case of Lys\(_{1041}\), with Pt(NO\(_2\))\(_4\)), we speculate that...
the effects of mutations at these sites on channel block are indirect; in other words, that mutations at these sites somehow alter the structure of the primary portal in a way that promotes suramin entry into and/or binding within this portal (Fig. 8C). If this is the case, then it may also be that the weakly charge-dependent or charge-independent effect of mutations (Fig. 6C) or chemical modifications (17) on Cl− conductance might also be ascribed to an indirect effect on Cl− entry via the primary portal (Fig. 8C). For example, one speculative mechanism by which this might take place is if these mutations lead indirectly to a widening of the primary portal, which could in theory facilitate the entry of large blocker molecules while at the same time impair entry of Cl− ions because of a reduction in the electrostatic influence of positive charges in the primary portal. Alternatively, we cannot rule out that Lys978, Lys1041, and/or Arg1048 directly influence the entry of a small amount of Cl− from the cytoplasm via a separate pathway (Fig. 8D) or that these residues lie in some recess that can be accessed from the primary portal that allows them to directly interact with Cl− ions but not with suramin (Fig. 8E). In either of these cases, the side chains of Lys978, Lys1041, and/or Arg1048 could contribute to the overall positive electrostatic potential that influences the movement of cytoplasmic Cl− ions into the pore. However, in either of these cases we would have to assume that mutation of these residues has both direct (electrostatic) and indirect (structural) effects on portal function, an assumption for which direct experimental evidence is lacking.

In conclusion, our functional results suggest that a single portal is sufficient to explain entry of cytoplasmic Cl− and blocking ions into the CFTR pore. This conclusion appears consistent with the reported structure of the TMEs (Fig. 8, A and B). Positive charges at Arg303 and Lys370 appear located close to the mouth of this portal (Fig. 8A), where they strongly impact suramin block (Fig. 3). Closer to the interior of the protein (Fig. 8, A and B), Lys190 and Arg303 strongly attract Cl− ions “upwards” toward the TM pore (Figs. 2 and 6), consistent with the important role proposed previously (17, 27). Suramin molecules may be too large to penetrate into the interior of the protein via this route. Around the periphery of the portal, Lys978, Lys1041, and Arg1048 may play minor roles in interactions with Cl− ions that, as described above, may be direct or indirect.

**Experimental procedures**

Experiments were carried out on baby hamster kidney cells transiently transfected with human CFTR, as described in detail previously (20). Mutant forms of CFTR were generated using the QuikChange site-directed mutagenesis system (Agilent Technologies, Santa Clara, CA) and verified by DNA sequencing. Functional characterization of wildtype and mutant CFTR channels was carried out using single channel and macroscopic patch-clamp recordings from inside-out membrane patches, as described in detail previously (20). Following 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 intracellular solution. As in earlier studies (19, 21, 22), single-channel currents were recorded after weak PKA stimulation (1–5 nM), whereas all macroscopic CFTR currents were recorded after maximal PKA.
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Figure 6. Effect of additional mutations on chloride conductance and suramin block. A, mean single channel current–voltage relationships for the glutamate-substituted channel mutants listed. Wildtype data as in Fig. 2B. B, mean fraction of control current remaining following addition of 10 μM suramin to the intracellular solution as a function of voltage in these channel variants. The means of data from 5–7 patches are shown. C, mean single channel conductance (measured from the slope of the current–voltage relationship from individual patches) as a function of side chain charge at the different positions listed. Black symbols represent wildtype (charge +1); charge 0 represents glutamine (Q) substitution at each site, and charge −1 glutamate (E) substitution. D, mean fraction of control current remaining following addition of 10 μM suramin to the intracellular solution (at −100 mV) for the same channel variants shown in C. E, mean single channel conductance as a function of the number of positively charged side chains neutralized by mutation to glutamine, in single mutants (change in charge −1) and double mutants (change in charge −2) as listed. F, mean fraction of control current remaining following addition of 10 μM suramin to the intracellular solution (at −100 mV) for the same channel variants shown in E. The means of data from 4–8 patches are shown.

Figure 7. Differential effect of mutations on Pt(NO$_2$)$_4$$^{2-}$ block. A, example macroscopic current–voltage relationships for different channel variants in inside-out patches. In each case currents were recorded before (control, black) and after (red) addition of 50 μM Pt(NO$_2$)$_4$$^{2-}$ to the intracellular solution. B, mean fraction of control current remaining following addition of this concentration of Pt(NO$_2$)$_4$$^{2-}$ as a function of voltage in these channel variants. The means of data from 5–7 patches are shown.
stimulation (~20 nM) and subsequent treatment with sodium pyrophosphate (2 mM) to stabilize the channel open state. As in previous studies of both single-channel conductance (17, 18, 23) and channel block (24 –26), the intracellular (bath) solution contained 150 mM NaCl, 2 mM MgCl₂, 10 mM TES, pH 7.4, whereas the extracellular (pipette) solution contained 150 mM sodium gluconate, 2 mM MgCl₂, 10 mM TES, pH 7.4, resulting in an outwardly directed Cl⁻ concentration gradient. Current traces were filtered at 100 (for macroscopic currents) or 50 Hz (for single channel currents) using an eight-pole Bessel filter, digitized at 250 Hz, and analyzed using pCLAMP10 software (Molecular Devices, Sunnyvale, CA). Measurement of single channel and macroscopic current amplitudes and construction of leak-subtracted macroscopic current–voltage relationships were carried out as described previously (20). Membrane voltages were corrected for liquid junction potentials calculated using pCLAMP software.

Block of macroscopic current by intracellular suramin and Pt(NO₂)₄²⁻ was investigated as described previously (13, 19, 22). Apparent blocker dissociation constant, $K_d$, was approximated from the following equation,

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

(Eq. 1)

where $[B]$ is blocker concentration, $I$ is current amplitude following the addition of blocker, and $I_0$ is the control, unblocked current amplitude. Suramin concentration–inhibition relationships (Fig. 4) were fitted by an equation that assumes a fixed fraction of the current is suramin-insensitive,

$$I/I_0 = [(1 - nh)/(1 + ([B]/K_d)^{nh})] + rf$$

(Eq. 2)

where $nh$ is the slope factor or Hill coefficient, and $rf$ is the suramin-resistant fraction of the current (0 –1).

**Figure 8. Models of cytoplasmic portal structure and function.** A and B, structure of the TMEs in zebrafish CFTR in a near-open state (8) (see also Fig. 1, D and E). Positively charged amino acids mutated in the present study are shown as space-filling models, viewed from above (A) or from the side (B; looking toward the portal entrance). In A, the red arrow indicates the entrance to the portal (note that no similar sized portal was observed on the “opposite” side of the TMEs (8)). In B, the block dotted line indicates the approximate extent of the apparent portal entrance. As noted in Fig. 1, the numbering of these positively charged amino acids is slightly different in zebrafish CFTR. C–E, models of portal function. C, Arg²⁴⁸ and Lys³⁵⁰ are proposed to lie close to the mouth of a single portal, with Lys¹⁹⁰ and Arg¹⁰⁴⁸ located more internally. All four of these positively charged residues attract cytoplasmic Cl⁻ (green) and suramin (red) electrostatically. Mutation of Lys⁹⁷⁸, Lys¹⁰⁴¹, and Arg¹⁰⁴⁸ affects the function of this portal at a distance, perhaps via propagated structural changes, to impact Cl⁻ and suramin entry indirectly. D, alternative to C, Lys⁹⁷⁸, Lys¹⁰⁴¹ and/or Arg¹⁰⁴⁸ could contribute to a second entry pathway for Cl⁻, whereas at the same time indirectly impacting suramin entry via the main portal. E, location of Lys⁹⁷⁸, Lys¹⁰⁴¹ and/or Arg¹⁰⁴⁸ in a “recessed” part of the pore might allow direct interaction with small Cl⁻ ions but not large suramin molecules. Again, an indirect impact on suramin entry would also have to be supposed.
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The experiments were carried out at room temperature, 21–24 °C. The values are presented as the means ± S.E. For graphical presentation of mean values, the error bars represent S.E., and where no error bars are visible, S.E. is smaller than the size of the symbol. Tests of significance were carried out using Student’s two-tailed t test, with p < 0.05 being considered statistically significant. All chemicals were from Sigma–Aldrich except for K_2Pt(NO_3)_4 (Strem Chemicals, Newburyport, MA) and PKA (Promega, Madison, WI).

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