Divergent CFTR orthologs respond differently to the channel inhibitors CFTRinh-172, glibenclamide, and GlyH-101

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Stahl M, Stahl K, Brubacher MB, Forrest JN Jr. Divergent CFTR orthologs respond differently to the channel inhibitors CFTRinh-172, glibenclamide, and GlyH-101. Am J Physiol Cell Physiol 302: C67–C76, 2012. First published September 21, 2011; doi:10.1152/ajpcell.00225.2011.—Comparison of diverse orthologs is a powerful tool to study the structure and function of channel proteins. We investigated the response of human, killifish, pig, and shark cystic fibrosis transmembrane conductance regulator (CFTR) to specific inhibitors of the channel: CFTRinh-172, glibenclamide, and GlyH-101. In three systems, including organ perfusion of the shark rectal gland, primary cultures of shark rectal gland tubules, and expression studies of each ortholog in cRNA microinjected Xenopus laevis oocytes, we observed fundamental differences in the sensitivity to inhibition by these channel blockers. In organ perfusion studies, shark CFTR was insensitive to inhibition by CFTRinh-172. This insensitivity was also seen in short-circuit current experiments with cultured rectal gland tubular epithelial cells (maximum inhibition 4 ± 1.3%). In oocyte expression studies, shark CFTR was again insensitive to CFTRinh-172 (maximum inhibition 10.3 ± 2.5% at 25 μM), pig CFTR was insensitive to glibenclamide (maximum inhibition 18.4 ± 4.4% at 250 μM), and all orthologs were sensitive to GlyH-101. The amino acid residues considered responsible by previous site-directed mutagenesis for binding of the three inhibitors are conserved in the four CFTR isoforms studied. These experiments demonstrate a profound difference in the sensitivity of different orthologs of CFTR proteins to inhibition by CFTR blockers that cannot be explained by mutagenesis of single amino acids. We believe that the potency of the inhibitors CFTRinh-172, glibenclamide, and GlyH-101 on the CFTR chloride channel protein is likely dictated by the local environment and the three-dimensional structure of additional residues that form the vestibules, the chloride pore, and regulatory regions of the channel.

shark rectal gland; Xenopus laevis oocytes; forskolin; isobutylmethylxanthine; two-electrode voltage clamp; cystic fibrosis transmembrane conductance regulator

Cystic fibrosis (CF) results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial chloride channel (25) that is expressed in secretory and absorptive epithelia in the airways, pancreas, intestine, testis, and other tissues. The disease is characterized by chronic lung infection, pancreatic insufficiency and male infertility, with progressive deterioration of lung function and death (42). CFTR is a member of the ATP-binding cassette family of membrane proteins (13) but is unique within this family in functioning as an ion channel rather than a transporter protein.1

CFTR is composed of two regions of six transmembrane domains (TMDs), two nucleotide-binding domains (NBDs), and a cytosolic regulatory region (R domain) that contains multiple sites for cAMP-dependent phosphorylation (1, 51). Transport of ions through pore-forming transmembrane α-helices is controlled by the NBDs, which interact with ATP to form a dimer (61). This ATP-driven dimerization of CFTR’s cytosplastic nucleotide-binding domains is directly linked to the opening of the ion channel in the transmembrane domains.

CFTR was cloned more than two decades ago (6, 45), but the atomic structure of the protein remains unclear as only low-resolution structures of CFTR are available (46, 65). Obtaining a high-resolution structure of CFTR holds promise for targeted therapy of CF.

Ion permeation through ion channels is influenced by charged amino acid side chains at the entrance of the channel pore (20). These residues attract oppositely charged ions from the solution, increasing their effective local concentration, while repelling ions of like charge (38, 53). Functional evidence suggests that permeant anions bind to several discrete sites within the CFTR channel pore (12, 30, 33, 54, 57). These binding sites attract chloride ions into the CFTR pore and coordinate ion-ion interactions that are necessary for rapid ion movement through the pore (17, 18). Site-directed amino acid mutagenesis studies implicate the positively charged amino acid side chains of K95 (14) and R334 (19, 53). R347 in TM6 may not interact directly with permeating anions but instead forms a salt bridge with D924, thus stabilizing the pore (11).

Inhibitors of the CFTR channel have been employed as tools to investigate the role of key amino acids in the CFTR channel pore. Chloride ion-binding sites within the CFTR pore serve as sites at which substances bind to occlude the pore and inhibit chloride permeation through the channel (15, 16, 37, 69). A diverse group of organic anions inhibit chloride transport by this mechanism (9, 23, 48). Those that have been studied extensively include the sulfonyleurea glibenclamide (50, 67, 69) and the glymine hydrizide GlyH-101 (39). Glibenclamide and GlyH-101 act as open channel blockers, glibenclamide blocking intracellularly and GlyH-101 extracellularly. Another well-studied inhibitor, the thiazolidone CFTRinh-172 (8, 32, 58, 59), does not function as an open channel blocker but rather affects channel gating (27). Despite several site-directed mutagenesis studies (8, 21, 29), the location and number of these inhibitor binding-sites in CFTR remain unclear.

1 This article is the topic of an Editorial Focus by David N. Sheppard (51a).
The three agents tested are not specific inhibitors of the CFTR channel. Glibenclamide inhibits other ATP-binding cassette (ABC) transporters including the sulfonylurea receptor and P-glycoprotein and calcium-activated Cl⁻ channels in mammalian cardiac myocytes (64). GlyH-101 inhibits other anion channels and transporters such as TMEM16A (10) and the SLC26 anion exchangers SLC26a3, -a6, a9, and -a11 (3, 56). CFTRinh-172 inhibits sodium transport in sweat glands (62). Both CFTRinh-172 and GlyH-101 affect mitochondrial function, independent of their action on CFTR (24).

Studies comparing protein orthologs have been a powerful tool for examining the structure and function of CFTR (31, 41, 43, 44). The evolutionary distance between orthologs, on one hand, and the conservation of certain motifs on the other, permit the study of structure-function relationships without site-directed mutagenesis and thus without manipulating the protein under examination. In this work we investigate the response of four different CFTR orthologs to multiple inhibitors of the channel. We investigated human- (45), killifish- (52), pig- (41), and shark CFTR (22, 34). Using three different systems, we observe fundamental differences among orthologs in the inhibition by different CFTR channel blockers.

MATERIALS AND METHODS

In vitro perfusion of shark rectal glands. Rectal glands were obtained from dogfish sharks, Squalus acanthias, of either sex weighing 2–6 kg that were euthanized by pithing using the approved short-circuit current (described in detail previously (7). Following stabilization of the basal gradient, the SLC26 anion exchangers SLC26a3, -a6, a9, and -a11 (3, 56). CFTRinh-172 inhibits sodium transport in sweat glands (62). Both CFTRinh-172 and GlyH-101 affect mitochondrial function, independent of their action on CFTR (24).

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MATERIALS AND METHODS

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Primary cell cultures of shark rectal gland tubules and measurements of transepithelial chloride transport as short-circuit current in primary culture monolayers: inhibition with CFTRinh-172, glibenclamide, and GlyH-101. Isolated tubules of shark rectal gland (SRG) epithelial cells were prepared under sterile conditions as previously described (60), and rectal gland tubules were plated onto Corning Transwell polyester membrane inserts (pore size 0.4 μm, membrane diameter 6.5 mm, catalog no. CLS3470, Sigma Aldrich, St. Louis, MO). Confluent primary culture monolayers were mounted in a modified Ussing chamber and bathed with a solution containing (in mM) 270 NaCl, 4 KCl, 3 MgCl₂, 2.5 CaCl₂, 8 NaHCO₃, 1 KH₂PO₄, 350 urea, and 5 glucose at pH 7.5. The chamber was kept at 20°C and was constantly gassed with 99% O₂ and 1% CO₂. The voltage-clamp and data acquisition equipment was designed and constructed by W. Van Driessche (Catholic University, Louvain, Belgium) and has been described in detail previously (7). Following stabilization of the basal short-circuit current (Isc), a steep apical-to-basolateral Cl⁻ gradient was imposed by replacing the basolateral perfusate by a solution containing (in mM) 270 Na-glucosamine, 4 K-glucosanote, 3 MgCl₂, 2.5 CaCl₂, 8 NaHCO₃, 1 KH₂PO₄, 350 urea, and 5 glucose at pH 7.5. Subsequently, nystatin was added basolaterally (0.72 mg/ml) to measure apical Cl⁻ absorption from a 1,000-fold concentrated stock solution in DMSO followed by brief sonication. After stabilization of

Fig. 1. In vitro perfusion of shark rectal glands. At 30 min, 1 μM forskolin and 100 μM IBMX were added to the perfusate in all glands. CFTRinh-172 (10 μM) was added from 50–70 min in experimental glands (n = 5), and the effect on chloride secretion was not statistically different compared with controls (n = 18). Values are mean chloride secretion in μeq h⁻¹ g⁻¹ ± SE.

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calculated using the current at the clamped voltage of $-20$ mV and the current at the clamped voltage of $+20$ mV. During the experiments, oocytes were perfused with ND96 containing 1.8 mM CaCl$_2$. I-V ramps were taken under basal conditions and during stimulation by forskolin (10 µM) and IBMX (1 mM). When the stimulation reached a steady state, the inhibitors were added, beginning with the lowest concentration. IBMX and forskolin was continually perfused during the different inhibitor concentrations. Once currents with one concentration of the inhibitor reached a steady state, the next higher concentration of the same inhibitor was used (Fig. 4). Identical concentrations of inhibitors were used on the four orthologs. All concentrations of inhibitors were greater than the IC$_{50}$ for human CFTR. The inhibitors examined were as follows: CFTRinh-172 (Sigma) and GlyH-101 (obtained from Cystic Fibrosis Foundation Therapeutics) at concentrations of 5, 10, and 20 µM and glibenclamide (Sigma) at concentrations of 50, 100, and 200 µM. Inhibition was determined by the ratio of the conductance measured at the steady state of forskolin/IBMX stimulation and the conductance obtained at the steady state of inhibition by the dose

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Fig. 2. Inhibition of short-circuit current ($I_{sc}$) by CFTR inhibitors in primary cultures of shark rectal gland (SRG) tubular epithelial cells. A: summary of mean % inhibition with 10 µM CFTRinh-172 ($n=8$), 300 µM glibenclamide ($n=3$), and 90 µM GlyH-101 ($n=4$). After stimulation with C-type natriuretic peptide (CNP) and forskolin, CFTRinh-172 inhibited to only $4.0 \pm 1.3\%$, glibenclamide to $54 \pm 7.42\%$, and GlyH-101 to $64 \pm 3.9\%$. The inhibition by glibenclamide and GlyH-101 is highly significant ($***P < 0.001$) compared with CFTRinh-172. Values represent means ± SE. B–D: representative $I_{sc}$ recordings with CFTRinh-172 (B), glibenclamide (C), and GlyH-101 (D).
of the specific inhibitor. Data were analyzed with pCLAMP software (Axon Instruments, Sunnyvale, CA). Results are expressed as microsiemens (µS) ± SE. P values were obtained using Student’s t-test, comparing the percent inhibition of the different orthologs to the percent inhibition of human CFTR at the same concentration of the inhibitor.

Dose-response experiments for CFTRinh-172, inhibition of sCFTR- vs. hCFTR-injected oocytes, and for glibenclamide, inhibition of pCFTR- vs. hCFTR-injected oocytes. Dose-response experiments for the most significant findings (sCFTR insensitivity to CFTRinh-172 compared with hCFTR and pCFTR insensitivity to glibenclamide compared with hCFTR) were performed by testing additional concentrations of each inhibitor. Those for CFTRinh-172 were 1, 5, 8, 10, 15, 20, and 25 µM and for glibenclamide were 20, 50, 80, 100, 150, 200, and 250 µM, reaching water solubility limits for both inhibitors with the maximum concentration used. IC50 values were calculated using the PRISM 5 software (GraphPad, La Jolla, CA).

All reagents and chemicals were purchased from Sigma Aldrich unless otherwise noted.

RESULTS

In vitro perfusion of SRGs. Experiments with CFTRinh-172 were first carried out in the perfused SRG. After 30 min of low basal chloride secretion, when the rectal gland is stimulated by forskolin (1 µM) + IBMX (100 µM), chloride secretion abruptly increased 15- to 30-fold from basal values of 100–250 µeq·h−1·g−1 to values of 2,400 to 3,000 µeq·h−1·g−1 (Fig. 1). In experiments where CFTRinh-172 (10 µM) was added from 50–70 min to the perfusate of stimulated glands receiving forskolin and IBMX, there was no effect of this channel blocker compared with control glands perfused with forskolin and IBMX only (Fig. 1).

Measurements of transepithelial chloride currents in SRG tubular epithelial cells: inhibition with CFTRinh-172, glibenclamide, and GlyH-101. Experiments with cultured monolayers of SRG epithelial cells confirmed the insensitivity of rectal gland chloride secretion to CFTRinh-172 observed in perfusion experiments (Fig. 2). Nystatin permeabilization of the basolateral membrane was applied to examine CFTR-mediated chloride currents driven by an inwardly directed Cl− concentration gradient across the apical membrane. After stimulation with CNP and forskolin, CFTRinh-172 inhibited to only 4.0 ± 1.3% (n = 8), glibenclamide to 54 ± 7.4% (n = 3), and GlyH-101 to 64 ± 3.9% (n = 4) of the stimulated current. (Fig. 2A): Representative tracings for each inhibitor are shown in Fig. 2, B–D.

Conductance of hCFTR-, kfCFTR-, pCFTR-, and scCFTR-injected oocytes and un.injected and water-injected controls under basal and stimulated conditions. Conductance of oocytes was measured in 122 oocytes injected with either hCFTR (n = 35), kfCFTR (n = 18), pcCFTR (n = 24), sCFTR (n = 21), un injected (n = 12) or water-injected (n = 12). hCFTR, kfCFTR, pCFTR, and sCFTR had basal conductances of 11.9 ± 1.4 µS, 20.5 ± 1.9 µS, 13 ± 3.3 µS, and 6.7 ± 1.3 µS, respectively. Uninjected and water-injected oocytes had lower baseline conductances of 7.2 ± 1.2 µS and 6.2 ± 0.6 µS (P < 0.05 compared with hCFTR) (Fig. 3, basal). Human-, shark-, and pig CFTR basal currents were under 5% of maximum stimulated currents. Killifish CFTR basal conductance was above 5% of maximum stimulated conductance and was significantly elevated compared with human CFTR, un injected, and water-injected oocytes (P < 0.001). Elevated basal chloride conductance has previously been observed in primary cultures of killifish opercular epithelium (35).

After addition of 10 µM forskolin and 1 mM IBMX to the perfusate, hCFTR, kfCFTR, pCFTR, and sCFTR had similar steady-state conductances (206 ± 21.9 µS, 218.7 ± 14.3 µS, 160.1 ± 29.5 µS, and 160.7 ± 34 µS, respectively; Fig. 3 max). However, in un injected and water-injected oocytes the conductances after stimulation with forskolin/IBMX were unchanged compared with baseline (5.9 ± 1.6 µS for un injected and 5.8 ± 0.5 µS for water injected) (both P < 0.001 compared with hCFTR after stimulation).

Time course of the CFTR inhibitors CFTRinh-172, glibenclamide, and GlyH-101 on the four CFTR orthologs in representative oocyte experiments. In each experiment (Fig. 4), oocyte chloride conductance increased as CFTR was stimulated by forskolin/IBMX. Three concentrations of each inhibitor were then added sequentially at time points indicated by the arrows. The mean conductance values for all studies with the three inhibitors in all orthologs are given in Figs. 5, 6, and 7. As illustrated in Fig. 4A, hCFTR was highly responsive to inhibition by CFTRinh-172, whereas pig and shark CFTR were insensitive to this inhibitor. In Fig. 4B, hCFTR and sCFTR were sensitive to increasing concentrations of glibenclamide, whereas killifish CFTR inhibition was intermediate and pig CFTR was insensitive to all concentrations of glibenclamide. In contrast (Fig. 4C), all four orthologs of CFTR were sensitive to the inhibitor GlyH-101.

Response to CFTRinh-172 in CFTR from diverse orthologs (hCFTR, kfCFTR, pCFTR, and sCFTR) in oocytes. We compared the response of the four orthologs of CFTR to increasing concentrations of CFTRinh-172 (5, 10, 20 µM) (Fig. 5). CFTRinh-172 inhibited hCFTR significantly at each concentration (65.5 ± 2.9% at 20 µM). However, we observed less response to CFTRinh-172 with kfCFTR and pCFTR. pCFTR inhibition was significantly less than human at all concentrations examined (P < 0.001 compared with hCFTR). At
the highest concentration (20 μM), inhibition of pCFTR was 20.6 ± 5.1% (Fig. 5A). In the \textit{Xenopus} oocyte expression system, similar to the perfused SRG and cultured tubular cells, sCFTR was practically unresponsive to CFTR\textsubscript{inh-172} (1.9 ± 0.9% inhibition at 5 μM, 4.9 ± 1.2% at 10 μM, 8.5 ± 1.5% at 20 μM). The failure of CFTR\textsubscript{inh-172} to inhibit sCFTR is highly significant at all concentrations compared with hCFTR (Fig. 5A). I-V relationships for hCFTR and sCFTR are shown in Fig. 5, B and C. I-V ramps were taken at the end of each condition: baseline perfusion with frog Ringer’s solution, stimulation with forskolin and IBMX, and inhibition with CFTR\textsubscript{inh-172}. The I-V plots illustrate that hCFTR and sCFTR show similar currents at baseline and after stimulation. However, they differ markedly in the currents seen after addition of CFTR\textsubscript{inh-172}.

Fig. 4. Representative recordings of conductance from \textit{Xenopus} oocytes expressing different CFTR orthologs. Recordings begin with addition of forskolin/IBMX (10 μM/1 mM) to stimulate conductance. The sequential application of the three increasing concentrations of each inhibitor is indicated by 1 for the lowest, 2 for the medium, and 3 for the highest concentration. A: forskolin/IBMX (10 μM/1 mM) and CFTR\textsubscript{inh-172} (5, 10, and 20 μM). B: forskolin/IBMX and glibenclamide (50, 100, and 200 μM). C: forskolin/IBMX and GlyH-101 (5, 10, and 20 μM).

Fig. 5. Comparison of the effects of CFTR\textsubscript{inh-172} on different CFTR orthologs. A: summary of mean % inhibition of hCFTR (n = 16), kfCFTR (n = 5), pCFTR (n = 4), and sCFTR (n = 12) with 5, 10, and 20 μM CFTR\textsubscript{inh-172}. The orthologs h-, kf-, and pCFTR were inhibited by CFTR\textsubscript{inh-172}, although pCFTR was much less responsive (20.6 ± 5.1% at 20 μM). However, sCFTR was insensitive to CFTR\textsubscript{inh-172} at all concentrations (1.9 ± 0.9% inhibition at 5 μM, 4.9 ± 1.2% at 10 μM, and 8.5 ± 1.5% at 20 μM). The lack of inhibition of sCFTR and pCFTR by CFTR\textsubscript{inh-172} is highly significant compared with hCFTR (P < 0.001 at all concentrations). Values are means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001 compared with hCFTR. B and C: representative current-voltage (I-V) plots from oocytes expressing hCFTR (B) and sCFTR (C). FOK, forskolin.
Response to glibenclamide in CFTR from diverse orthologs (hCFTR, kCFTR, pCFTR, and sCFTR). Glibenclamide inhibited hCFTR significantly (32.6 ± 2.8% inhibition at 50 μM, 48 ± 3.1% at 100 μM, and 65.8 ± 3.6% at 200 μM) (Fig. 6). Unlike the response to CFTRinh-172, sCFTR was responsive to glibenclamide (26.6 ± 4.8% inhibition at 50 μM, 35.1 ± 8.5% at 100 μM, and 44.6 ± 9.4% at 200 μM) (Fig. 6). Similar to the results with CFTRinh-172, kCFTR was also less responsive to glibenclamide at higher concentrations (30.2 ± 6.7% at 200 μM, P < 0.001 compared with hCFTR).

Response to glyh-101 on different CFTR orthologs (hCFTR, kCFTR, pCFTR, and sCFTR). Glyh-101 on different CFTR orthologs (hCFTR, kCFTR, pCFTR, and sCFTR). Glyh-101 on different CFTR orthologs (hCFTR, kCFTR, pCFTR, and sCFTR).
The most striking observation with glibenclamide was the markedly impaired response of pCFTR to this inhibitor (6.8 ± 1.2% inhibition at 50 μM, 11.2 ± 1.8% at 100 μM, and 15.2 ± 3.0% at 200 μM) (Fig. 6A). Glibenclamide was unable to inhibit conductance in oocytes injected with pCFTR compared with hCFTR (P < 0.001 for each concentration of inhibitor). The I-V plots for hCFTR and pCFTR are shown in Fig. 6, B and C. Despite similar basal and stimulated currents, hCFTR was significantly inhibited by glibenclamide (200 μM), while pCFTR remained practically unchanged.

**Response to Gly-H-101 in CFTR from diverse orthologs.** In contrast to CFTRinh-172 and glibenclamide, hCFTR, kfCFTR, scCFTR, and pCFTR were each responsive to Gly-H-101 (Fig. 7). At the higher concentrations of Gly-H-101 used (10 and 20 μM), inhibition of p- and scCFTR was significantly greater than hCFTR (P < 0.001 for each compared with hCFTR). The I-V plots for hCFTR and sCFTR are shown in Fig. 7, B and C. Despite similar levels of current before and after stimulation with IBMX/forskolin, sCFTR was much more inhibited by 20 μM of Gly-H-101 compared with hCFTR (P < 0.001). In agreement with Muanprasat et al. (39), we observed inward rectification of hCFTR currents at lower concentrations of Gly-H-101 (5 and 10 μM) but not at higher concentrations (20 μM).

**Dose response to CFTRinh-172 in scCFTR** vs. hCFTR-injected oocytes and to glibenclamide in pCFTR vs. hCFTR-injected oocytes. We carried out further dose-response experiments and calculated IC_{50} values for the most significant findings of our study (scCFTR insensitivity to CFTRinh-172 and pCFTR insensitivity to glibenclamide). In oocytes expressing hCFTR, the dose response with CFTRinh-172 revealed an IC_{50} of 10.4 μM and a maximal inhibition of 73.3 ± 7.4%. In contrast, in scCFTR-injected oocytes, maximal inhibition by CFTRinh-172 was only 10.3 ± 2.5% with a theoretical IC_{50} of 250 μM (Fig. 8A). For glibenclamide, in oocytes expressing hCFTR, dose-response experiments displayed an IC_{50} of 102 μM and a maximal inhibition of 74.4 ± 4.2%. However, pCFTR-injected oocytes showed maximal inhibition of only 18.4 ± 4.4% with a theoretical IC_{50} of 930 μM (Fig. 8B).

**DISCUSSION**

In summary, our experiments demonstrate the following: 1) The thiazolidone inhibitor CFTRinh-172 does not inhibit shark CFTR in any of the three preparations examined, including homologous expression in the intact perfused shark rectal gland, primary monolayer cultures of rectal gland epithelial cells, and heterologous expression in *Xenopus* oocytes. In the oocyte, CFTRinh-172 does inhibit human, pig, and killifish CFTR, although this significance is significantly less in pig CFTR compared with humans. 2) In the oocyte, the sulfonamide glibenclamide does not inhibit pig CFTR. Furthermore, there was no change in the magnitude of block of kCFTR over the concentration range studied. 3) The glycine hydrazide Gly-H-101 is a universal inhibitor of CFTR, blocking the channel in all four orthologs, but with inhibition of the pig and shark protein being significantly greater than human.

These experiments examined well-established inhibitors of CFTR (36, 48), using comparison of four wild-type orthologs rather than mutagenesis to explore the charged vestibule model of the CFTR protein. Green and Andersen (20) envisioned the general features of channel structure as evolutionary solutions to the limitations on conduction imposed by diffusion (20). They suggested that rapid ion translocation across membranes is enhanced if the narrow region of the channel pore, responsible for selectivity and binding, is flanked by vestibules designed to increase the capture radius at the channel mouth and to concentrate permeant ions using electrostatic forces. This model is widely used to explain structure-function relationships of CFTR (49, 53) and consists of three parts: an outer vestibule, pointing towards the outside of the cell membrane; a narrow region, representing the rate limiting selectivity filter; and an inner vestibule that opens to the cytoplasm. Since CFTR transports negatively charged chloride ions, positively charged amino acids adjacent to the rate limiting section of the pore are thought to enhance chloride permeation (36).

The three negatively charged inhibitors studied are proposed to bind at different sites of the channel, Gly-H-101 to the outer vestibule and glibenclamide to the inner vestibule (49). CFTRinh-172 does not function as an open channel blocker since there is no rectification of current during block (32). Several different mechanisms of block have been proposed (24, 27).
Multiple site-directed mutagenesis studies have been performed to elucidate specific binding sites of the CFTR inhibitors. Caci et al. (8) proposed R347 as important for blockade of CFTR by CFTRinh-172. Gupta et al. (21) observed that two mutations in the 6th transmembrane region, F337A and T338A, significantly weakened glibenclamide block. Linsdell et al. (29) identified K95 as an important residue in the channel pore inner vestibule for binding of glibenclamide. St. Aubin et al. (55) identified R303, a second important residue of the inner vestibule region. They conclude that larger open channel blockers might bind to R303, whereas smaller molecules can penetrate deeper into the pore and bind to K95.

For GlyH-101, no site-directed mutagenesis studies have been performed. Since GlyH-101 block shows inward rectification, it is likely that GlyH-101 binds in the external pore vestibule (39). Several residues in the external mouth of the CFTR pore have been proposed as anion binding sites; however, none have been examined for binding of GlyH-101. R334 (15, 18), R104, R117, and K335 (68) have been suggested to be important for binding of GlyH-101 by homology modeling and glibenclamide by mutagenesis. Norimatsu et al. (40) used homology modeling of CFTR to identify potential GlyH-101 binding sites F337 and T338, which were previously found in mutagenesis studies to be important for glibenclamide block (21).

When the amino acid sequence alignment of the four CFTR orthologs is examined (Fig. 9), we found that each of the residues described above is conserved in all four orthologs of CFTR. We emphasize that this conservation of residues includes both those altered in mutagenesis studies for CFTRinh-172 and glibenclamide binding and those proposed in homology modeling for GlyH-101. Thus, these single amino acids alone cannot explain the variations in response to inhibitors seen in our studies.

Considering that R347 alone does not explain the striking differences in CFTRinh-172 potency among different CFTR orthologs, we conclude, in agreement with Caci et al. (8), that the binding site of CFTRinh-172 likely lies elsewhere in the CFTR protein and that R347 has primarily an allosteric effect. Indeed, the R347D mutation alters ATPase activity in the NBDs, suggesting that R347 might be involved in conformational coupling between the TMDs and the NBDs (26). Furthermore, the observation of fundamental differences in the response to CFTRinh-172 in CFTR orthologs sharing high similarity in sequence and function, in the same expression system, argues that CFTRinh-172 binds directly to the CFTR protein and does not act primarily on subcellular structures like mitochondria as proposed previously (24). Our results offer new information favoring a complex mechanism of CFTRinh-172 inhibition with the inhibitor acting directly on the channel.

The insensitivity of pCFTR to glibenclamide observed by Liu et al. (31) in primary cultures and in our oocyte experiments also cannot be explained by differences in single amino acid residues between the orthologs. There are two conflicting mechanisms proposed for the glibenclamide block of conductance (21). Single inhibitor binding sites (47, 50) and multiple binding sites (21, 36, 66) have been proposed. Our results are consistent with the later model favoring a complex mechanism of block involving multiple binding sites.

Given the many differences between orthologs observed in these experiments that cannot be explained by targeting single amino acids, we conclude that study of wild-type channels from diverse species offers critical new information. We believe that the potency of the inhibitors CFTRinh-172, glibenclamide, and Gly-H101 on the CFTR chloride channel protein is dictated by the three-dimensional structure of additional residues.
dues that contribute to the inhibitor binding sites, the vestibules, and the chloride channel pore.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

M.S., K.S., M.B.B., and J.N.F. conceived and designed the research; M.S., K.S., and M.B.B. performed experiments; M.S., K.S., and J.N.F. analyzed data; M.S., K.S., M.B.B., and J.N.F. interpreted results of experiments; M.S., K.S., and M.B.B. prepared figures; M.S., K.S., M.B.B., and J.N.F. drafted manuscript; M.S., K.S., and J.N.F. edited and revised manuscript; M.S., K.S., M.B.B., and J.N.F. approved final version of manuscript.

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