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REVIEW

PERMEATION THROUGH THE CFTR CHLORIDE CHANNEL

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Summary

The cystic fibrosis transmembrane conductance regulator (CFTR) protein forms a Cl^- channel found in the plasma membranes of many epithelial cells, including those of the kidney, gut and conducting airways. Mutation of the gene encoding CFTR is the primary defect in cystic fibrosis, a disease that affects approximately 30 000 individuals in the United States alone. Alteration of CFTR function also plays an important role in the pathophysiology of secretory diarrhea and polycystic kidney disease. The basic mechanisms of permeation in this channel are not well understood. It is not known which portions of the protein contribute to forming the pore or which amino acid residues in those domains are involved in the biophysical processes of ion permeation. In this review, I will discuss (i) the present understanding of ion transport processes in the wild-type CFTR channel, (ii) the experimental approaches currently being applied to investigate the pore, and (iii) a proposed structure that takes into account the present data on mechanisms of ion selectivity in the CFTR channel and on blockade of the pore by open-channel blockers.

Key words: cystic fibrosis, cystic fibrosis transmembrane conductance regulator, CFTR, open-channel blocker, anion selectivity.

Introduction

Cystic fibrosis (CF) is the most common lethal, autosomal recessive inherited disease among Caucasians, affecting approximately one in 3000 live births in the USA. It is the result of mutations in a single gene (Riordan et al., 1989) that encodes a large membrane protein called the cystic fibrosis transmembrane conductance regulator (CFTR). This protein is expressed in multiple tissues of epithelial origin, where it has been given a number of putative functions, the most accepted of which is in the formation of a Cl^- transport pathway. In each of these tissues, CFTR forms a Cl^- channel that is responsible for establishing the appropriate transcellular movement of salts and water into or from the luminal spaces of these tissues, although the specific role of CFTR in the maintenance of the ionic composition and the volume of airway surface fluid remains controversial (Zabner et al., 1998; Matsui et al., 1998). Although the disease affects several tissues and organ systems (including the pancreas, the intestine, the sweat duct and the reproductive tract), the most debilitating clinical manifestations of CF are found in the lung as a result of the loss of this secretory pathway in the epithelial cells of the conducting airways. The disrupted balance in the ionic and osmotic composition of the airway surface fluid leads to an inability to fight off colonization by bacteria that are tropic for airway cells (i.e. *Pseudomonas* species) and to entry into a vicious cycle of infection and inflammation that eventually leads to death as a result of the loss of respiratory tissue (Quinton, 1999). CFTR is also involved in two other diseases of note: polycystic kidney disease and secretory diarrhea, including cholera. Activation of CFTR and the basolateral cotransporter in cholera leads to life-threatening dehydration (Gabriel et al., 1994). In polycystic kidney disease, cyst activating factor stimulates both cell proliferation and Cl^- secretion, the latter *via* activation of CFTR and the basolateral cotransporter (for a review, see Sullivan et al., 1998).

Structure of the CFTR protein

Work in the early 1980s showed that the basic defect in tissues affected by CF was the loss or misregulation of a Cl^- current in the apical membranes of these epithelial cells. Hence, when the defective gene was cloned in 1989, it came as no surprise that the protein product was predicted to form an integral membrane protein with 12 amphipathic transmembrane (TM) helices (Riordan et al., 1989), much as one would expect for an ion channel protein. However, CFTR was also predicted to have a secondary structure novel to ion channels: there are three large putatively cytoplasmic domains with primary sequences not found in any other ion channel to date. The overall structure proposed consists of five functional domains: two hydrophobic membrane-spanning domains

(MSD1, MSD2; Fig. 1), each including six TM helices; two hydrophilic membrane-associated domains containing sequences that form nucleotide-binding domains (NBD1, NBD2); and a regulatory (R) domain that carries multiple consensus sequences for phosphorylation by protein kinases A (PKA) and C (PKC). This secondary structure places CFTR in the ATP-binding cassette (ABC) transporter superfamily of proteins (Higgins, 1992, 1995). Members of this superfamily catalyze the membrane transport, in an ATP-dependent manner, of a wide variety of substrates ranging from small inorganic ions and metabolites to large hydrophobic drugs and polypeptides. In each case, substrate transport is achieved in a manner that is obligatorily linked to the binding and hydrolysis of ATP. The gating process in CFTR is likewise dependent upon ATP hydrolysis (Anderson et al., 1992; Baukrowitz et al., 1994; Hwang et al., 1994; Zeltwanger et al., 1999; Gadsby and Nairn, 1999; Csanády and Gadsby, 1999).

However, CFTR is unique within this group, because it functions as an ion channel. Although the density of CFTR expression is highest in epithelial cells, PKA-activated Cl^- currents have been observed in mammalian cardiac ventricular myocytes for many years (Harvey and Hume, 1989; Bahinski et al., 1989; Harvey et al., 1990; Nagel et al., 1992; Horowitz et al., 1993; Gadsby et al., 1995). It is believed that this channel is a splice variant of CFTR in which the product of the fifth exon is deleted, resulting in a shortening of the first cytoplasmic loop (Fig. 1). Myocytes of nearly all non-primate mammals studied express the exon-deleted transcript (Horowitz et al., 1993; Hume and Horowitz, 1995), but the functional significance of the loss of these 30 amino acid residues, particularly regarding effects on permeation, is not yet known (Yamazaki et al., 1999). Cardiac CFTR may play a role in the regulation of action potential duration and in establishing the resting potential in myocytes in which it is expressed (Gadsby et al., 1995; Hume and Horowitz, 1995).

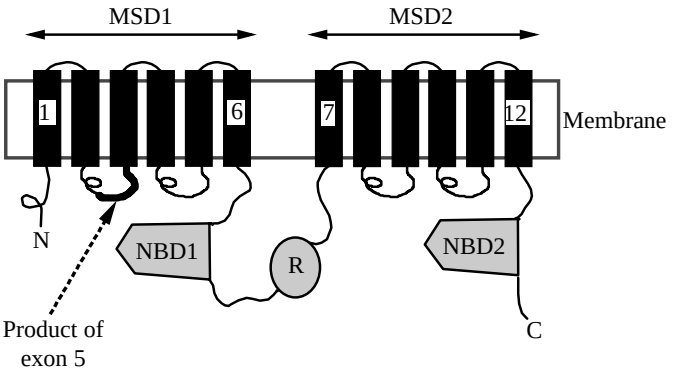

Fig. 1. Structural model of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CFTR is a large integral membrane protein, predicted to have both amino and carboxy termini localized in the cytoplasm. Membrane-spanning domains (MSDs) 1 and 2 include six putative transmembrane domains each. The cytoplasmic gating complex consists of two hydrophilic nucleotide-binding domains (NBD1 and NBD2) plus the regulatory (R) domain. The cardiac form of the CFTR gene transcript lacks exon 5, which results in the deletion of 30 amino acid residues from the first cytoplasmic loop.

The CFTR gene sequences of several non-human species have also been determined (rhesus monkey, Wine et al., 1998; mouse, Tata et al., 1991; rat, Fiedler et al., 1998; cow, Diamond et al., 1991; sheep, Tebbutt et al., 1994; dogfish, Marshall et al., 1991; yeast, Miosga and Zimmerman, 1998; rabbit, cardiac form, Horowitz et al., 1993; Yamazaki et al., 1999; frog, Tucker et al., 1998; killifish, Singer et al., 1998). Only these last three, however, have been assembled into full-length, expressible clones. Given that most of the published sequences for CFTR variants cannot be linked to biophysical properties of expressed channels, one must approach the idea of identifying conserved domains with some wariness. Nonetheless, comparison of these sequences shows that the degree of conservation at the amino acid level is quite high overall and strikingly high in certain domains. Mutations that cause CF disease in patients are not usually informative for our present purpose because most of these lead to proteins that are not fully processed into and positioned in the plasma membrane (Cheng et al., 1990; Welsh and Smith, 1993).

CFTR is both an ion channel and a regulator of channels

This review focuses on the Cl^- channel function of CFTR. However, there has been much speculation about other functions for CFTR. Given that most other members of the ABC transporter superfamily move their substrates in a manner more consistent with transporter-like behavior, much effort has been spent in trying to identify substrates other than Cl^- that may be transported by CFTR. The question remains unanswered, at this point. However, heterologous expression studies have shown that CFTR is *at least* a Cl^- channel. Expression of CFTR cDNA in a variety of cells leads to the appearance of a channel whose characteristics match those of one of the cyclic-AMP-activated Cl^- permeabilities defective in CF patients (Welsh, 1990; Cliff et al., 1992). Nonetheless, CFTR is a multifunctional protein, with at least two roles in epithelial cell membranes. In addition to its intrinsic ion channel activity, CFTR also regulates the activity of several other types of ion channel (for reviews, see Higgins, 1995; Schwiebert et al., 1999), including ORCC Cl^- channels (Schwiebert et al., 1995), ENaC Na^+ channels (Stutts et al., 1995), ROMK2 K^+ channels (McNicholas et al., 1996, 1997) and Kir6.1 K^+ channels (Ishida-Takahashi et al., 1998), although the mechanisms by which it does so are not clear (Schwiebert et al., 1998).

Permeation through the wild-type CFTR channel

What do we know about the basics of permeation in the wild-type (WT) CFTR channel? Human CFTR exhibits a low single-channel conductance, typically 8–10 pS at room temperature in the presence of 150 mmol l^{-1} Cl^-. WT-CFTR currents exhibit no voltage- or time-dependence, over steps ranging from 1 ms to several seconds to potentials between

–100 and +80 mV. Anion channels usually discriminate imperfectly between anions and cations (Dawson et al., 1999). CFTR provides no exception to this generality: these channels typically show only a 10-fold preference for Cl^- over Na^+ (Anderson et al., 1991). WT-CFTR currents in the presence of symmetrical [Cl^-] as the sole anion exhibit linear current–voltage (*I*/*V*) relationships. Outward rectification (non-linearity of the *I*/*V* relationship, indicating outward currents enhanced above inward currents at any potential difference around the reversal potential) can be induced in excised patches expressing CFTR by the inclusion of large impermeant anions in the cytoplasmic bathing solution (Overholt et al., 1993, 1995; Linsdell and Hanrahan, 1996b). Recent studies have shown that CFTR allows permeation of large organic anions when present at the intracellular but not the extracellular side of an excised patch; this asymmetry is dependent upon ATP hydrolysis (Linsdell and Hanrahan, 1996b, 1998a,b). Thus, there is a distinct possibility that the processes of gating and permeation are linked in CFTR, as appears to be the case in several other channels (Khakh and Lester, 1999), such that the permeation properties of the pore are dependent upon the conformational state induced by the gating process. Two other pieces of evidence suggest that this may be the case for CFTR. First, block of the channel pore by MOPS occurs at a rate that is dependent upon whether the channel is in a highly activated state or a poorly activated state (Ishihara and Welsh, 1997). Second, we have described a pore-domain mutation in CFTR that confers voltage-jump relaxations in the macroscopic current and also shortens the open time of single channels (Zhang et al., 2000a). Most interestingly, the rate of the macroscopic relaxation depends upon the character of the permeating anion, suggesting a link between gating and permeation in this CFTR variant.

One of the parameters that describes a channel to a particularly high degree of detail is its ability to select between ions of similar charge. This functional distinction arises from a structural arrangement that is finely tuned to provide this specification; an example is found in the recent elucidation of the crystal structure of a K^+ channel (Doyle et al., 1998). Ion selectivity is very sensitive to pore structure because it reflects the three general steps in permeation of an ion through the channel: *solvation* at the mouth of the pore, when the ion is at least partially dehydrated and is stabilized by interaction with some portion of the pore walls; *translocation*, as the ion moves between binding sites within the pore; and *desolvation* or rehydration as the ion moves into the bulk solution as it exits the pore (see Fig. 2) (Hille, 1992). The processes of solvation and desolvation, then, occur at rates that are dependent upon the differences between ion–water and ion–pore interaction energies. By studying the rates of these processes in the absence of a membrane potential, one defines the permeability of the channel to a given ion. In practice, however, the permeability of the substitute ion *relative* to that of a standard ion is calculated as described below, generating values for *relative permeability* rather than absolute permeability. The process of translocation is highly dependent upon the driving force for anion permeation and reflects the strength of interaction between the anion and each binding site. Anions that bind tightly exhibit a reduced rate of current flow, or conductance, through the channel because of their longer dwell-time at one or more binding sites. In practice, the conductance for a substitute ion relative to that of a standard ion is determined either from the unitary conductance in single channels or from whole-cell conductances, as described below, to calculate the *relative conductance*. For a mathematical treatment of these concepts, the reader is directed to a recent review by Dawson et al. (1999).

Patterns of selectivity vary widely and can tell us much about the structural features of a given channel. However, ‘selectivity’ is a general term that can be misused: it only has meaning when a distinction is made between processes that involve permeability and processes that involve conduction. We think about anion selectivity as being defined in multiple ways. Selectivity based upon ion size occurs through size effects on hydration energies and size effects causing physical occlusion from the pore. Some anions exhibit a reduced conductance because they bind tightly. Anion channels such as CFTR are selective for monovalent anions over divalent anions. Some ions exhibit anomalous behavior: examples for CFTR are I^- and ClO_4^-. Finally, anion channels also have mechanisms that make them selective for anions over cations. A complete picture of selectivity requires a broad set of assays in structure/function experiments.

Several modern theories attempt to explain ion selectivity in structural and energetic terms. In one useful framework, ion selectivity depends upon the relative magnitudes of the energy required to dehydrate the ion and the energy gained by interaction with the pore. As noted above, two measures are typically used for these studies: relative permeability and relative conductance. Relative permeabilities reflect the ease with which a channel protein can pull an ion from solution into the ‘capture volume’ within the pore vestibule and, hence, may be highly dependent upon the hydration energy for each given ion (Eisenman and Horn, 1983; Dawson et al., 1999). A relative permeability sequence indicating a low-affinity ion–pore interaction would be $I^->Br^->Cl^->F^-$. A high-affinity interaction would result in a relative permeability sequence of $F^->Cl^->Br^->I^-$. For small anions, WT-CFTR has a sequence of $Br^->Cl^->I^->F^-$ (Tabcharani et al., 1997; Zhang et al., 2000a), which suggests that permeation in CFTR may involve a combination of low and high field strength interactions. Relative conductances, in contrast, are thought to be related to the affinity of the pore walls for a given anion so that ‘sticky’ anions that bind better than Cl^-, such as SCN^- and ClO_4^-, exhibit a reduced conductance (Dawson and Smith, 1997; Mansoura et al., 1998; Dawson et al., 1999). In terms of Eyring rate theory (Eyring et al., 1949), relative permeabilities relate to the heights of energy barriers for different anions while relative conductances relate to the depths of the wells in these energy diagrams (Fig. 2A) (Wright and Diamond, 1977; Eisenman and Horn, 1983; Dawson et al., 1999). While the use of rate theory, or any other model-dependent approach, to

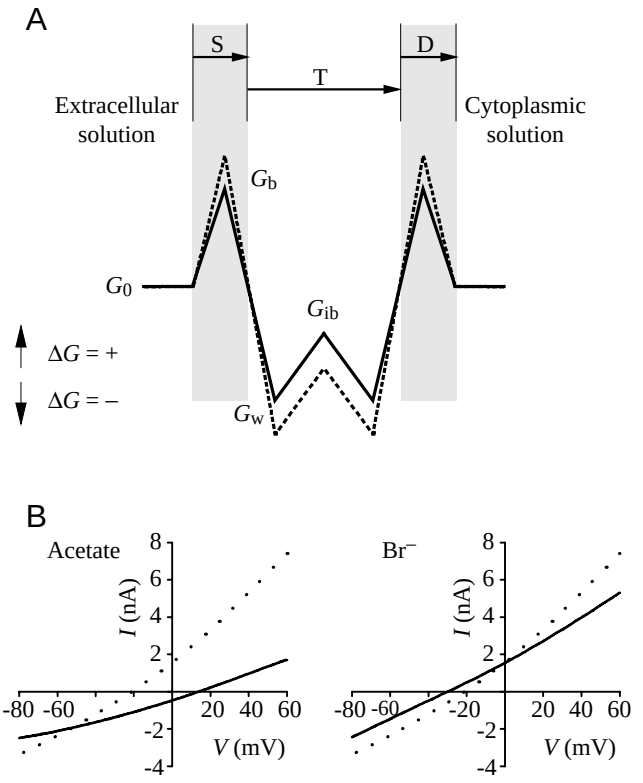

Fig. 2. Theoretical and practical descriptions of ion selectivity. (A) Schematic description of ion permeation, using barrier diagrams to describe the changes in free energy (ΔG) that an anion experiences as it transits the pore. An anion moving through the channel from left to right undergoes the three processes described in the text: solvation (S), translocation (T) and desolvation (D). Moving from the baseline energy state (G_0), the ion experiences an energetic barrier to its access to the pore (G_b) and then associates with a binding site, or well (G_w), at which the energy difference favors stabilization for some brief dwell time. For the sake of simplicity, the channel is modeled as a symmetrical three-barrier, two-site system in which the sites are separated by an inner barrier (G_{ib}), and with G_0 in the cytoplasm being equivalent to G_0 in the extracellular solution. The solid line indicates the energetic trajectory for a standard ion, such as Cl^-. The dashed line represents the trajectory for a test ion, such as acetate. (B) Example of a selectivity experiment. Current–voltage plots generated from two-electrode voltage-clamp currents for one oocyte expressing the wild-type cystic fibrosis transmembrane conductance regulator (WT-CFTR) in solutions containing Cl^- and two substitute anions are shown. The membrane potential was ramped between +60 mV and –80 mV over 200 ms; similar data are obtained using a voltage step protocol instead of voltage ramps. In each plot, the dotted line represents data collected in a solution containing Cl^- as the predominant anion. The solid lines represent data collected in a solution containing acetate (left) or bromide (right) as the predominant anion.

describe the process of diffusion of ions through the pore of channels has clear limitations (Miller, 1999; McCleskey, 1999), these concepts provide frameworks for experimentally testing hypotheses that may be useful. Using rate theory, Hanrahan and colleagues have characterized the CFTR channel as a multi-ion pore because of the presence of anomalous mole fraction effects in mixtures of Cl^- and SCN^- (Tabcharani et al., 1993). In their hands, relative permeability sequences for WT-CFTR suggest the presence of a weak field strength site (or sites) predominantly determined by residues in the sixth transmembrane domain (TM6) (Tabcharani et al., 1997; Linsdell et al., 1997b). The physical basis for this selectivity pattern, the 'lyotropic' selectivity pattern (Wright and Diamond, 1977), has recently been described by Dawson and coworkers (Smith et al., 1999). Block of WT-CFTR by gluconate was used to suggest that the pore can be modeled as one with three or four barriers and two or three wells (Linsdell et al., 1997a). Hence, we would predict that there are multiple ion-binding sites within the volume of the pore. This may help to explain the apparent complexity of selectivity patterns in WT-CFTR.

Relative permeabilities (P_X/P_{Cl}) are determined from the reversal potentials (E_{rev}) of whole-cell or single-channel currents in the presence of Cl^- (E_{rev}^{Cl}) or anions substituted for Cl^-. The Goldman–Hodgkin–Katz equation is used to calculate P_X/P_{Cl}. In whole-cell experiments, with anion substitutions made in the external bathing solution, a shift in reversal potential to a potential less negative than E_{rev}^{Cl} indicates a P_X/P_{Cl} less than unity for the substitute anion 'X', while a shift in reversal potential to a potential more negative than E_{rev}^{Cl} indicates a P_X/P_{Cl} greater than unity for the substitute anion (Fig. 2B). Relative conductances (G_X/G_{Cl}) are also determined from whole-cell or single-channel currents. In our whole-cell experiments, we calculate the chord conductance for anion entry by determining chord conductance over a range of E_{rev} to E_{rev} + 25 mV (Zhang et al., 2000a). A decrease in chord conductance in the presence of substituting anion 'X', compared with currents in the presence of Cl^-, indicates G_X/G_{Cl} values less than unity (Fig. 2B). The use of the Goldman–Hodgkin–Katz equation to estimate relative permeabilities implicitly assumes that the behavior of one ion is independent of the behavior of another ion or of the occupancy of the channel by any ion. This assumption fails for CFTR, and for any other channel with multiple ion-binding sites, because tight binding of one anion would be expected to increase the mean occupancy of the binding sites in the pore and, thereby, to decrease the permeability of another anion. However, this approach is intuitively useful and has been widely used for assessing selectivity in a broad range of channels (Dawson et al., 1999).

Table 1 summarizes P_X/P_{Cl} and G_X/G_{Cl} for a wide variety of substitute anions (McCarty and Zhang, 1998; Zhang et al., 2000a). In our experiments, macroscopic currents in *Xenopus laevis* oocytes expressing CFTR were measured using two-electrode voltage-clamp. Similar results have been described by others for CFTRs expressed endogenously in epithelial cells or heterologously in a number of systems (Anderson et al., 1991; Linsdell et al., 1997a,b, 1998; Tabcharani et al., 1997; Mansoura et al., 1998; Illek et al., 1999). For most anions, P_X/P_{Cl} is determined by the relative hydration energies of the ions, as expected for a channel with a lyotropic selectivity

Table 1. *Anion selectivity in the wild-type CFTR Cl^- channel*

Ion	E_{rev} (mV)	P_X/P_{Cl}	G_X/G_{Cl}	Radius (nm)	$\Delta_{hydr}G°$ (kJ mol^{-1})
SCN^-	–54.54	2.44	0.21	0.213	–287
NO_3^-	–38.28	1.30	0.93	0.189	–306
Br^-	–32.68	1.08	0.76	0.196	–321
Cl^-	–30.31	1.0	1.0	0.181	–347
I^-	–9.71	0.41	0.29	0.220	–283
Acetate	2.14	0.26	0.60	0.232	–373
Glutamate	7.11	0.22	0.61	0.275	–
Isethionate	8.69	0.18	0.48	0.260	–
ClO_4^-	9.32	0.17	0.17	0.240	–214
Gluconate	14.46	0.16	0.58	0.290	–

Relative permeability (P_X/P_{Cl}) and relative conductance (G_X/G_{Cl}) were determined from macroscopic measurements in oocytes expressing the wild-type CFTR Cl^- channel (WT-CFTR).

Ions are listed in order of decreasing relative permeability.

Values listed for ionic radii are the effective radii reported by Marcus (1997), except those for glutamate (from Franciolini and Nonner, 1987) and gluconate (from Halm and Frizzell, 1992).

Values for the change in Gibbs free energy upon hydration ($\Delta_{hydr}G°$) are also from Marcus (1997).

Experimentally determined values are from Zhang et al. (2000a).

E_{rev}, reversal potential.

sequence. Two anions, I^- and ClO_4^-, exhibit relative permeabilities lower than expected from this relationship. P_{ClO_4}/P_{Cl} is lower than expected for a weak field strength site, suggesting that the pore may contain a mixture of sites of varying field strength. Currents in the presence of external iodide indicate protocol- and voltage-dependent block of CFTR by this anion (Tabcharani et al., 1997). Relative permeabilities to isethionate, glutamate and gluconate are very low, as if these ions are too large to enter the pore. Relative conductances are also determined by ion size (Table 1). In our experiments on WT-CFTR, no anion exhibited a conductance for anion entry greater than that of Cl^-. Because the substitution solutions contain 4 mmol l^{-1} residual Cl^-, as well as 96 mmol l^{-1} substitute anion, we always observed a low baseline conductance in the presence of substitute anions; in no case did we measure a zero conductance. This allows us to separate the substitute anions into three classes with respect to conductance for anion entry in WT-CFTR: (i) anions that exhibit significant conductance (e.g. Cl^-, NO_3^-, Br^-); (ii) anions that are too large to fit easily into the pore (e.g. acetate, glutamate, gluconate, isethionate); and (iii) anions that are small enough to fit in, but bind so tightly that they block the current generated by the residual Cl^- (e.g. SCN^-, I^-, ClO_4^-). Hence, I^-, ClO_4^- and SCN^- exhibit paradoxically low values of G_X/G_{Cl}, indicating tight interactions between these anions and binding sites within the pore.

Experimental tools for studying pore structure

Given this background of data characterizing the wild-type channel, what tools can we use to identify the portions of the protein that confer these biophysical features? While structure/function studies of anion channels are still in their youth compared with similar studies of cation channels, the latter provide a rich history of ideas that can be applied to the former. Obviously, each of the parameters comprising the biophysical signature of CFTR (single-channel conductance, rectification, selectivity) can be compared between wild-type and site-directed mutants. Studies of this sort have identified amino acids that contribute to anion-binding sites and to sites that play direct or indirect roles in determining anion selectivity (see below). The method of ‘cysteine-scanning’ mutagenesis adapted to ion channels by Karlin and colleagues (Akabas et al., 1992) can be used to identify amino acid residues that have at least transient access to the water-filled volume of the pore. In this approach, cysteine residues are substituted individually for residues in putative pore-lining domains. The ability of an inserted cysteine to react with small water-soluble sulfhydryl-modifying reagents is taken as evidence that the cysteine in question has access to the pore and may, therefore, be involved in forming the pore itself. Some investigators feel that this method provides advantages beyond standard mutagenesis. However, there are many assumptions in this technique that have not been fully satisfied. Most importantly, it is essential to show that the reagents used interact with each engineered cysteine residue with appropriately rapid kinetics and in a manner that is modulated by the concentration of permeating anion. Another important caveat is that this technique relies on the assumptions (i) that the effects of modification observed in whole-cell experiments reflect changes in conduction properties of the channel, without affecting gating properties, and (ii) that the engineered cysteines that react with the water-soluble reagent do so because they line the water-filled permeation pathway, rather than lining a water-filled crevice in the folded protein. For instance, Horn and coworkers (Yang et al., 1996, 1997) showed that cysteine residues engineered into S4 segments of a voltage-dependent Na^+ channel were accessible from either side of the membrane. Modification of these cysteine residues produced effects on gating but not conduction. So, the S4 segment appears to exist in a water-filled ‘channel’, separate from the conduction pathway. This study also emphasizes the importance of studying the consequences of mutations at the single-channel level to distinguish between effects on gating and permeation.

A less popular method for structure/function studies of channels relies on the production of synporins, synthetic peptides with sequences derived from individual transmembrane helices of the channel of interest, which may form ion channels when reconstituted into lipid bilayers (Montal et al., 1990; Oblatt-Montal et al., 1994; Marsh, 1998). It is assumed that those sequences that form functional channels, preferably with a biophysical character resembling that of the full-length channel under similar conditions, correspond to sequences that contribute to the pore in the full-length channel.

Pharmacology of CFTR

Another approach to structure/function experiments in ion channels, termed 'reverse pharmacology', makes use of pharmacological agents that block the pore (Lester, 1988, 1991; Leonard et al., 1988). Pore-blocking drugs are used as probes of the pore structure by assessing the effects of mutagenesis upon blocking behavior. The method is so named because a drug of known structure is used to identify unknown structures within the channel pore, in contrast to the structure/activity relationships inherent to classical pharmacology. The key requirement of this approach is that one must prove that the drug of interest blocks the channel by an interaction within the pore itself, rather than through an allosteric interaction elsewhere in the channel that results in inhibition. In this regard, one can make use of what often amounts to a large amount of pharmacological information in the literature. Agents of a variety of types have served as tools for the identification and study of the pores of ion channels. High-affinity probes of K^+ channels, in the form of peptides such as charybdotoxin, enabled detailed analysis of the pore (MacKinnon and Miller, 1989; Gross and MacKinnon, 1996); and organic molecules, such as tetraethylammonium (TEA^+), also served as probes, albeit with lower affinity (MacKinnon and Yellen, 1990; Yellen et al., 1991). Information from both types of probe was verified when a K^+ channel pore-forming subunit was crystallized and studied at high resolution (Doyle et al., 1998). Unfortunately, peptide blockers of CFTR have not been described. No known organic blockers exhibit an affinity for CFTR as high as that of the peptide blockers of K^+ channels. Nonetheless, we have shown that organic blockers may be used to identify portions of the CFTR protein that contribute to the pore (McDonough et al., 1994; S. Zeltwanger, Z.-R. Zhang and N. A. McCarty, in preparation; Zhang et al., 2000b; Z.-R. Zhang, S. Zeltwanger and N. A. McCarty, in preparation). A comprehensive review of the pharmacology of CFTR has recently been published (Schultz et al., 1999).

Blockade of heterologously expressed CFTR by three classes of compound has been described: disulfonic stilbenes, the arylaminobenzoates and sulfonylurea compounds. Block by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) only occurs when the drug is applied to the cytoplasmic side of the membrane; this charged divalent anion is unable to cross the membrane by diffusion and cannot access its binding site from the outside (Linsdell and Hanrahan, 1996a). Mutation R347D at the putative cytoplasmic end of TM6 reduced the affinity for DIDS. However, R347D has recently been shown to grossly perturb the conformation of the pore by disruption of a salt bridge (Cotten and Welsh, 1999). Hence, the location of the DIDS-binding site is not established. Hanrahan and coworkers (Linsdell and Hanrahan, 1999) have also recently provided preliminary evidence that CFTR is blocked by substrates of the multidrug-resistance-related protein (MRP), a relative of CFTR in the ABC transporter superfamily. However, the mechanism is not known.

The arylaminobenzoates represent one of the most heavily studied classes of blockers of the CFTR Cl^- channel, but their

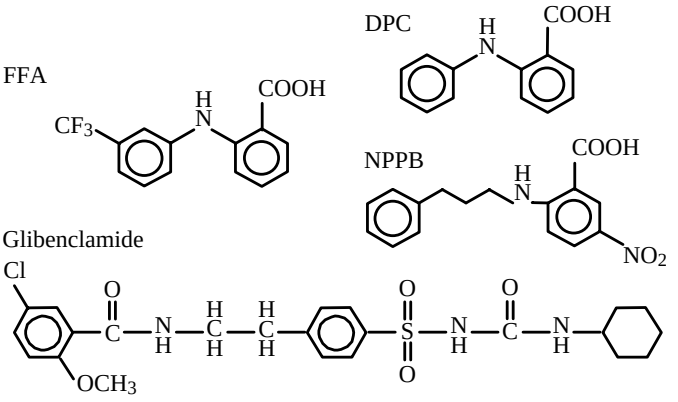

Fig. 3. Cl^- channel blockers that we use as probes of the pore in the cystic fibrosis transmembrane conductance regulator. DPC, diphenylamine-2-carboxylate; FFA, flufenamic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate.

mechanism of action remained unclear until recently. Voltage-dependent block of CFTR by diphenylamine-2-carboxylate (DPC), flufenamic acid (FFA) and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) (Fig. 3) has now been studied (McCarty et al., 1993; McDonough et al., 1994; Walsh and Wang, 1998; Walsh et al., 1999; Zhang et al., 2000b). In cardiac cells and in oocytes expressing wild-type epithelial CFTR, DPC and NPPB block CFTR with a similar voltage-dependence (Walsh and Wang, 1996, 1998; Zhang et al., 2000b).

We have now investigated block of WT-CFTR by these three arylaminobenzoates (DPC, FFA and NPPB) at the single-channel level (McCarty et al., 1993; McDonough et al., 1994; Zhang et al., 2000b). All three drugs block by accessing their binding sites from the cytoplasmic side of the channel and inhibit single channels at hyperpolarizing membrane potentials with simple kinetics. In each case, single-channel recordings in the absence of drug exhibit an endogenous intraburst closed time of brief duration (approximately 0.3 ms) that probably represents block by the pH buffer in the cytoplasmic solution (McCarty et al., 1993; Tabcharani et al., 1997). Application of drug led to the appearance of a longer closed state, during which occupancy of the drug at its binding site interrupted the flow of Cl^- current (Fig. 4). The drug-induced closed times increase in duration in the same order as the potency for inhibition of macroscopic currents: 0.62 ms, 1.11 ms and 2.35 ms for DPC, FFA and NPPB, respectively. Hence, we know that increased potency in this family arises at least in part from a decrease in the off-rate. In our whole-cell experiments, block of macroscopic currents is used to calculate the apparent K_D at each voltage and the voltage-dependence of block, as described by Woodhull (1973) and Zhang et al. (2000b). In side-by-side comparisons under identical conditions, block by DPC and block by NPPB exhibit nearly identical voltage-dependencies, suggesting a common binding site. Woodhull analysis indicated that these drugs experience approximately 38 % of the voltage field across the membrane, measured from the cytoplasmic side (Zhang et al., 2000b). Block of the pore

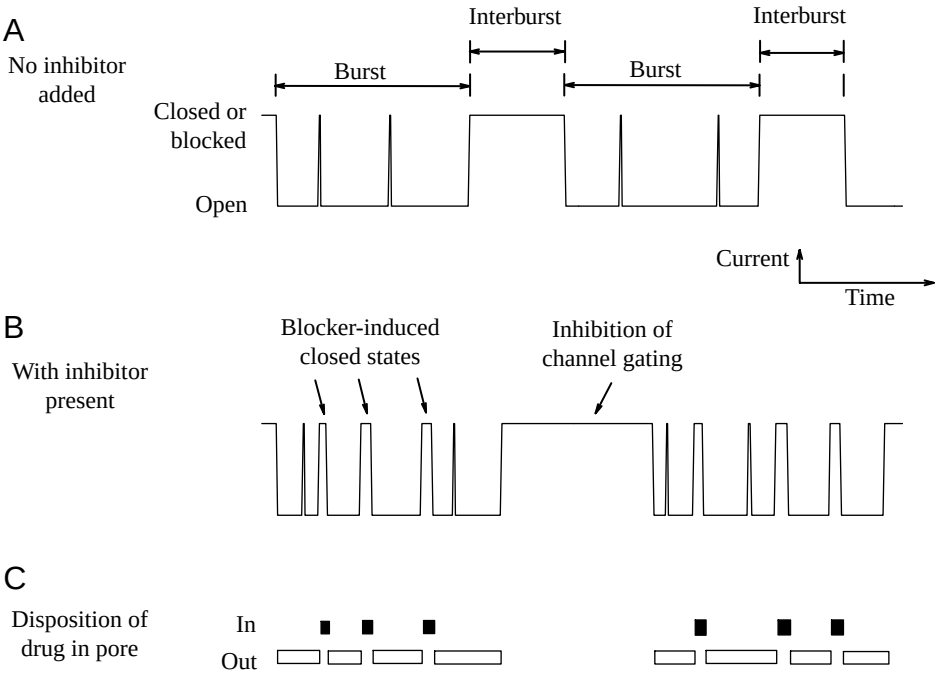

Fig. 4. Schematic description of open-channel blockade of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels in an excised, inside-out patch. (A) Idealized current trace in the absence of exogenously added blocker, under normal stimulating conditions. The channel opens for a period of hundreds of milliseconds (a burst). During this burst, brief transitions to the closed state represent block of the pore by the pH buffer TES, used in the intracellular solution. The time between bursts (interburst duration) is dependent upon the level of stimulation. The inverse of the interburst duration represents the channel opening rate. (B) Idealized current trace in the presence of a mixed-type inhibitor, such as glibenclamide, in the intracellular solution. In addition to the brief transitions to the closed state caused by the pH buffer, much longer closed states are evident in the presence of the blocker. During these blocker-induced closed states, ionic current is transiently interrupted by the presence of the blocking molecule in the pore, as shown in the time course represented in C. As a consequence of open-channel blockade, the duration of the burst is lengthened by the sum of the durations of the blocker-induced closed states. Mixed-type inhibitors may also interfere with channel gating, leading to an increase in the interburst duration (or decrease in opening rate), as shown in B.

by these drugs is rapid, leading to time-independent macroscopic currents; relief of the block is also rapid. A dose–efficacy relationship for DPC block of CFTR currents at −100 mV was fitted best with a Hill coefficient near unity, confirming that CFTR has only one DPC-binding site. We assume that this is also true for other arylaminobenzoates. Furthermore, the presence of a single class of drug-induced closed time suggests a single binding site for the arylaminobenzoates. These results are consistent with a mechanism of action by simple pore blockade. The interactions of DPC and NPPB with their binding site in WT-CFTR are pH-dependent: low bath pH alters the steady-state voltage-dependence (Zhang et al., 2000b).

Other data also support a pore-blocking mechanism. (i) The rapid flicker induced in CFTR single-channel recordings by exposure to NPPB (Zhang et al., 2000b) or DPC (McCarty et al., 1993) resembles open-channel block described in other systems (Neher and Steinbach, 1978; Hille, 1992), in which the flicker arises from the residence of the blocker at its site (Fig. 4). (ii) Blockade is voltage-dependent; the direction of the voltage-dependence is consistent with a requirement that the drugs access their binding sites after permeating the membrane to reach the cytoplasmic side of the channel, as shown by block of single channels in cell-attached mode after application of DPC to the extracellular medium (McCarty et al., 1993). (iii) Blockade is modulated by permeant anions, such that the efficacy of blockade is increased by a reduction in $[Cl^-]_o$ (McDonough et al., 1994) or reduced by substitution of Cl^- by SCN^- as the permeant anion (Walsh et al., 1999). (iv) NPPB significantly increases the open-burst duration in a dose-dependent manner (Zhang et al., 2000b), indicating that NPPB is a classical open-channel blocker in that the channel cannot close to the prolonged interburst closed state when the drug occupies its binding site (Fig. 4). (v) Finally, kinetic analysis of blockade by NPPB or DPC indicated that the mean intraburst open-time for single CFTR channels was inversely related to drug concentration, while mean intraburst closed time was unaffected (Zhang et al., 2000b). These findings strongly support the idea that the arylaminobenzoates block within the pore and, therefore, may be useful as probes of pore structure.

The sulfonylureas, including glibenclamide, are a class of hypoglycemic agents that are used to control the release of insulin from pancreatic β-cells by interaction with the sulfonylurea receptor, SUR, a member of the ABC transporter superfamily (Aguilar-Bryan et al., 1995). Glibenclamide also interacts with another member of the ABC transporter superfamily, P-glycoprotein, leading to the reversal of multidrug resistance (Golstein et al., 1999). Glibenclamide interacts with CFTR to modulate both the regulatory and ion-channel functions of this ABC transporter. With regard to the regulatory function, coexpression of CFTR and ATP-regulated K^+ channels (Kir6.1) confers glibenclamide-sensitivity to the K^+ channels (Ishida-Takahashi et al., 1998). CFTR is also capable of conferring glibenclamide-sensitivity upon ROMK2 K^+ channels expressed in oocytes (McNicholas et al., 1996, 1997) and upon ORCC Cl^- channels expressed endogenously in Hi-5 insect cells (Julien et al., 1999).

Inhibition of CFTR channel function by glibenclamide was first described by Sheppard and Welsh (1992). WT-CFTR and several variants expressed in mammalian cell lines were studied using whole-cell recording. Glibenclamide and its congener tolbutamide blocked CFTR with no apparent voltage-dependence. Schultz et al. (1996) studied the effects of glibenclamide on the bursting kinetics of CFTR in excised inside-out patches in mouse L cells. These authors described a decrease in burst duration and a decrease in interburst duration in the presence of 25 μmol l^{-1} glibenclamide. Because of the relatively high channel activity, they could not distinguish open-to-blocked from open-to-closed transitions. Voltage-dependence was not studied (Schultz et al., 1996). The same group found that tolbutamide blocked CFTR with a mechanism similar to that of glibenclamide (Venglarik et al., 1996). Other authors have shown that inhibition of Cl^- channel activity by glibenclamide is not unique to CFTR (Rabe et al., 1995). Swelling-activated Cl^- currents in cardiac myocytes are also inhibited by glibenclamide (Sakaguchi et al., 1997), as are Ca^{2+}-activated Cl^- channels (Yamazaki and Hume, 1997).

The first detailed kinetic analysis of the blockade of CFTR by glibenclamide was provided by Sheppard and Robinson (1997). The open-probability (P_o) of single WT-CFTR channels stably expressed in C127 cells decreased in a dose-dependent manner. Glibenclamide caused an increase in burst duration in some patches and a decrease in others. A single glibenclamide-induced intraburst closed time (mean duration approximately 15 ms) was observed, suggesting a single binding site for glibenclamide in CFTR, which experiences approximately 48 % of the membrane voltage field (Sheppard and Robinson, 1997).

We have extensively characterized the interaction between glibenclamide and WT-CFTR channels, using both whole-cell and single-channel measurements of CFTR expressed in *Xenopus laevis* oocytes (S. Zeltwanger, Z.-R. Zhang and N. A. McCarty, in preparation; Z.-R. Zhang, S. Zeltwanger and N. A. McCarty, in preparation). Glibenclamide blocks CFTR in a complex manner by interaction with multiple binding sites with varying affinity, voltage-dependence and pH-dependence. Both the on-rate and the off-rate for glibenclamide block of macroscopic CFTR currents in oocytes are much slower than those of other known CFTR blockers, resulting in time-dependent relaxations in the presence of the drug. At hyperpolarizing membrane potentials, block by 100 μmol l^{-1} glibenclamide developed over a time course of tens of milliseconds, resulting in substantial relaxations in the current trace. Following steps to depolarizing potentials, currents increased, again over a time course of tens of milliseconds. Relaxations at both hyperpolarizing and depolarizing potentials were fitted best with a second-order exponential function, which suggests the presence of two glibenclamide-binding sites influencing the pore. Interestingly, Woodhull analysis of inward currents indicated that the voltage-dependence of blockade changed during the relaxation. If the glibenclamide-induced relaxations are, as we proposed, due to time-dependent interactions between glibenclamide and two or more binding sites, this observation suggests that these binding sites differ in location within the membrane electric field.

Single-channel measurements (S. Zeltwanger, Z.-R. Zhang and N. A. McCarty, in preparation) indicated that glibenclamide blocks CFTR by interacting with three sites: two sites that appear to lie in the pore, and one site that may reside in one of the cytoplasmic gating domains. In excised patches, glibenclamide increased burst duration (consistent with an open-channel mechanism of block) and increased interburst duration (consistent with inhibition of ATP-dependent channel gating). The two pore-domain sites differ greatly in their kinetics. One has a fast on-rate but low affinity; the other has a slow on-rate but high affinity. The differences in the on-rates and affinities for these two states can explain the time-dependent behavior of macroscopic blockade in whole cells. It is not yet known whether binding to these sites differs in voltage-dependence. Finally, DPC and glibenclamide interact in the pore of CFTR, as shown by the following results in the simultaneous presence of the two blockers: (i) the macroscopic relaxation at depolarizing potentials is slowed nearly twofold (Z.-R. Zhang, S. Zeltwanger and N. A. McCarty, in preparation), and (ii) the microscopic off-rate from one of the two pore-domain sites is reduced nearly twofold (S. Zeltwanger, Z.-R. Zhang and N. A. McCarty, in preparation). Hence, DPC appears to inhibit the dissociation of glibenclamide from one of its binding sites. These data suggest that the cytoplasmic vestibule of the CFTR channel is large because the pore can accommodate both glibenclamide and DPC at the same time as Cl^-.

Defining the pore of the CFTR channel

In the remainder of this review, we will discuss progress towards determining which portions of the CFTR protein comprise the pore-lining domains and which residues within those domains play important roles in establishing the biophysical character of open CFTR channels. We will begin by discussing studies that address large domains, and then focus on the function of specific residues in individual TM domains.

Over the 10 years since the cloning of the human *CFTR* gene, progress has been made in defining portions of the protein comprising pore-lining domains and residues within those domains that play important roles in establishing the biophysical character of open CFTR channels (Sheppard and Welsh, 1999). Welsh and colleagues used chimera studies to show that differences between *Xenopus laevis* and human CFTR were governed by MSD1 (Price et al., 1996). However, there are only very minor differences between the human and *Xenopus laevis* versions of TM11 and TM12, so it may be that all the important residues in MSD2 are the same in these two species. Also, the limited assay used may be insufficient for showing other functional differences that may be determined by MSD2. Regional deletion mutants have also suggested that the sequences in TM1–TM6 are sufficient for formation of the pore, but function may require dimerization of the half-channel

(Sheppard et al., 1994; Schwiebert et al., 1998). Others have found that the N terminus of CFTR, including TM1–TM4, could be deleted without loss of function (Carroll et al., 1995). A splice variant similar to this is expressed in the kidney (Morales et al., 1996). Hence, it was thought that TM5–TM6, plus all of MSD2, may be the minimum component for function. More recent studies have shown that channels may be constructed from only the C-terminal half of CFTR, although these channels were not selective between Cl^- and I^- (Devidas et al., 1998). Mutagenesis studies also suggested that the portions of CFTR that function in Cl^- conduction were separate from the portions contributing to regulation of ORCC (Schwiebert et al., 1998).

How many TM domains contribute to the walls of the conduction pathway? The typical model for voltage-gated channels suggests four subunits, although this is not consistent amongst other channels. There has been no unequivocal evidence that CFTR pores require two or more subunits, except in cases where truncation mutations were expressed. Recombinant CFTR expressed in a variety of cells appeared to exist in monomeric form (Marshall et al., 1994). Concatamerized constructs expressed in HEK-293 cells show that cytoplasmic regulatory domains from two CFTRs can interact, although it was not clear that both constructs contributed to only one pore (Tao et al., 1996; Zerhusen et al., 1999). Freeze-fracture studies also suggest that heterologously expressed CFTR may be found in the membrane as dimers (Eskandari et al., 1998), although this method cannot distinguish between models with one or two pores. Dimerization of HisP, the ATP-binding subunit of the histidine permease, appears to be required for function (Nikaido et al., 1997; Hung et al., 1998). However, because HisP is equivalent to only one of the NBDs of CFTR, this information is consistent with interaction between the two NBDs of CFTR, not between two CFTR monomers. Structural analysis of one ABC transporter (P-glycoprotein, at 2.5 nm resolution) has been reported. Lectin-gold labeling of the single glycosylation site in P-glycoprotein particles indicated a monomeric form (Rosenberg et al., 1997). Nonetheless, the possibility exists that the functional CFTR channel is constructed from a dimer of CFTR peptides (Devidas and Guggino, 1997). A dual-pore model has even been suggested by computer modeling (Gallet et al., 1998), although this was performed without much regard to the large body of physiological data available.

The first structure/function studies in CFTR tested charge-reversal mutations in TM1, TM6 and TM10 (Anderson et al., 1991); mutations at two of the four residues studied resulted in modest changes in halide selectivity. These authors concluded that TM1 and TM6 contributed to the pore. Synthetic peptides with sequences of TM2 and TM6, but not others in MSD1, produced Cl^--selective channels when incorporated into lipid bilayers (Montal et al., 1990; Oblatt-Montal et al., 1994). Akabas et al. (1994) used cysteine-scanning mutagenesis to study TM1; they identified a surface of the TM1 helix that appears to line the pore (although this was not supported by studies at G91 in this domain; Mansoura et al., 1998) and have now extended that work to TM6 and TM3 (Cheung and Akabas, 1996, 1997; Akabas, 1998).

The majority of work investigating individual amino acid residues in the pore of CFTR has emphasized residues in or flanking TM6, which has received such extensive attention simply because it is the helix that includes a greater number of charged amino acids than any other. Here, it is believed that K335 and R347 influence selectivity and Cl^- conductance (Tabcharani et al., 1997): R347 confers anomalous mole-fraction effects and protocol-dependent block by I^- (Tabcharani et al., 1997); cysteine substitutions for several residues interact with SH-modifying reagents as if they face the pore (Cheung and Akabas, 1996); T338 and T339 together control the permeability of the channel to polyatomic anions as if they contribute to a narrow region (Linsdell et al., 1997b, 1998); disease-associated mutations (R334W and R347P) alter the kinetics and conductance of single channels (Sheppard et al., 1993); and the anion/cation selectivity filter is formed by T351, R352 and Q353 (Cheung and Akabas, 1997; Guinamard and Akabas, 1999). Fig. 5 shows the sequences of TM5, TM6, TM11 and TM12, displayed as helical nets. Each circle represents one amino acid residue. Red circles indicate residues for which investigations using various methods (other than cysteine-scanning mutagenesis) have indicated a potential role for this amino acid in forming the surface with which anions interact. The angle of this collection of noted residues along the length of the TM6 helix implies that the helix may be tilted with respect to the membrane, assuming that the pore is a cylinder running perpendicular to the membrane surface. It is important to note that all studies attributing a functional role to R347 must be reconsidered, because substitution of this residue with anything other than lysine or histidine grossly disrupts the conformation of the channel (Cotten and Welsh, 1999). For details of experimental findings in TM6, the most highly studied TM in CFTR, we refer the reader to an interactive figure that can be found at the following URL: http://www.emory.edu/WHSC/MED/PHYSIOLOGY/NMCC/JEB.html.

Since it is unlikely that the pore of this putatively monomeric channel protein is constructed from a single TM helix, other domains must also contribute amino acids to the pore. Dawson and coworkers have suggested that TM5 may contribute to the pore on the basis of the finding that mutations at G314 and V317 in this domain alter conduction properties (Mansoura et al., 1994, 1998; Smith et al., 1997). Evidence that TM11 and TM12 also contribute to the pore comes both from selectivity studies and from an analysis of the effects of mutations in these domains upon interactions with open-channel blockers (McDonough et al., 1994; McCarty and Zhang, 1999; Zhang et al., 2000a,b).

We have shown that residues in TM6 supply most of the binding energy for blockade by DPC and NPPB, and that residues in TM11 and TM12 also contribute to the binding site for these drugs. Mutations at S341 in TM6, S1118 in TM11 (Zhang et al., 2000a) and T1134 in TM12 altered the affinity and/or voltage-dependence of blockade by DPC or NPPB

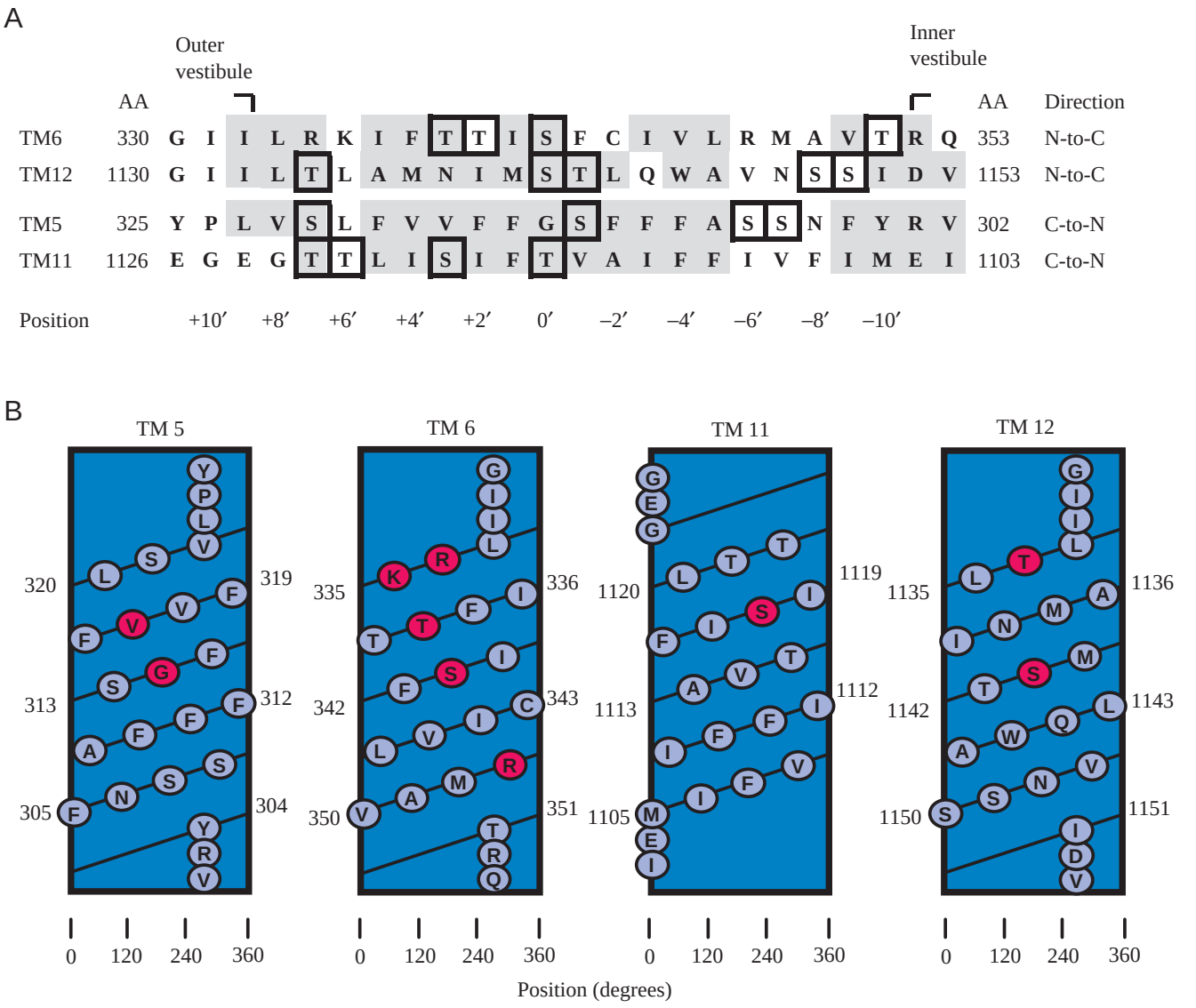

Fig. 5. Identification of transmembrane (TM) domains that may contribute to the pore of the cystic fibrosis transmembrane conductance regulator (CFTR). (A) Proposed alignment of residues in TM helices 5, 6, 11 and 12. Note that these sequences are written such that their intracellular and extracellular ends are aligned as they would be in the folded protein. Hydroxylated residues are boxed. Shading indicates regions of similar amino acid (AA) character. Below the alignment is the numbering system we propose for residues in these TM domains. S341 is positioned at the 0′ position, because mutations at this position have extensive effects on permeation properties. (B) Sequences of TMs 5, 6, 11 and 12 of CFTR shown as helical nets. The amino acid sequence of each TM is displayed as if the cylinder of the helix has been sliced open along its long axis, which allows the displacement of adjacent positions by 100 ° to be visualized. Note that we do not know the absolute boundaries of the helices at their extracellular and intracellular ends. The numbers along the left and right sides of the boxes indicate the position of the adjacent amino acid. Circles in red indicate residues that have been shown to be important biophysical determinants of permeation in CFTR, by a number of assays. The alignment of these residues, at least in TM6, suggests a surface that may form one wall of the pore. An interactive version of the TM6 helical net, which lists the experimental results concerning each amino acid shown here, can be found at the following URL: http://www.emory.edu/WHSC/MED/PHYSIOLOGY/NMCC/JEB.html. This figure will be maintained for 1 year from the date of publication.

(McDonough et al., 1994; Zhang et al., 2000b). Mutations at S341 and T1134 also affected blockade by glibenclamide, suggesting that the binding pockets for these drugs overlap (Z.-R. Zhang, S. Zeltwanger and N. A. McCarty, in preparation). Mutations at several positions in TM6 affect biophysical parameters such as single-channel conductance, rectification and anion selectivity (McDonough et al., 1994; Linsdell et al., 1997b, 1998; McCarty and Zhang, 1998, 1999). Our data suggest that K335 (in TM6) and T1134 (in TM12) play an indirect role in determining selectivity. In fact, they may lie just at the innermost edge of the outer vestibule, rather than in the pore itself. In contrast, mutations at T338 in TM6 have extensive effects on anion selectivity, suggesting that this position contributes to a region of high discrimination in the

pore (Linsdell et al., 1998; McCarty and Zhang, 1999). S341, in TM6, appears to lie in the most critical portion of this region of high discrimination, because mutations here affect single-channel conductance, selectivity among monovalent anions, monovalent/divalent anion selectivity, anion/cation selectivity and sensitivity to block by DPC, NPPB and glibenclamide (McDonough et al., 1994; McCarty and Zhang, 1999; Zhang et al., 2000b; Z.-R. Zhang, S. Zeltwanger and N. A. McCarty, in preparation). Whether this region of high discrimination extends further towards the cytoplasmic end of the pore is not known.

A three-dimensional picture is emerging

Many studies from a variety of laboratories have identified the critical role that TM6 plays in determining the permeation properties of CFTR (McDonough et al., 1994; Linsdell et al., 1997a). However, this one-dimensional view is very limited in its ability to describe the environment within the pore and cannot account for all the experimental data. The anions that permeate the pore and the drugs that block it are three-dimensional molecules and will probably interact with portions of the pore contributed by multiple TM domains. Hence, it will be important to consider the structure of the pore in three dimensions, taking into account the contributions made by amino acid residues in TM domains other than TM6.

Our working hypothesis is that the pore of CFTR is lined by residues contributed by TM domains 5, 6, 11 and 12 (Fig. 5). We previously modeled TM6 and TM12 by their homology to ligand-gated anion channels (McDonough et al., 1994). It is clear that TM6 and TM12 are not mirror images of each other. Hence, the pore of CFTR is asymmetric, in contrast to the pores of ligand-gated channels and many voltage-gated channels (Lester, 1992). Inspection of the sequences for TM5 and TM11 shows similar patterns of hydroxylated and hydrophobic residues when TM5 and TM11 are written inverted so that the extracellular ends of these four domains are aligned (Fig. 5). TM11 is surprisingly similar to TM6 and even more so to TM12, including the repeated motif of hydroxylated residues every 3–4 positions and adjacent to bands of hydrophobic groups. TM5, in contrast, is less amphipathic. A goal of current studies in my laboratory is to determine which amino acid residues in TM5, TM11 and TM12 lie in positions homologous to the positions of amino acid residues in TM6. The lower portion of Fig. 5A includes a numbering system that we hope will facilitate discussion of the positions within each helix (McCarty and Zhang, 1999) and will allow comparisons of positions between multiple helices. A similar system has proved useful in the field of ligand-gated channels (Lester, 1992). We position the 0′ point at a location equivalent to S341 in TM6, since our results show this residue to be so critical to conduction, selectivity and blockade (McDonough et al., 1994; McCarty and Zhang, 1999; Zhang et al., 2000b; Z.-R. Zhang, S. Zeltwanger and N. A. McCarty, in preparation).

Taking into consideration the results from many laboratories, a somewhat fuzzy picture is emerging. We

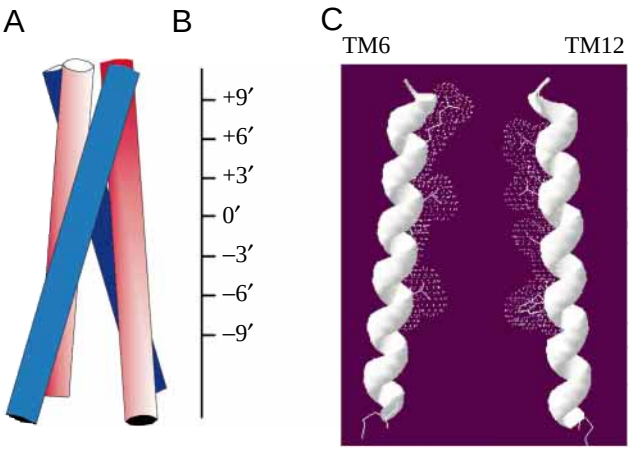

Fig. 6. Three-dimensional model of the pore in the cystic fibrosis transmembrane conductance regulator (CFTR). (A) Proposed orientation of four α-helices, transmembrane (TM) domains 5, 6, 11 and 12. The TM domains cross each other at some distance from the extracellular end (at top). We envision TM6 and TM12 lying across the pore from each other (red or blue pair), and TM5 and TM11 forming the other pair. (B) Vertical scale indicating the relative positions of residues in these TM domains using the proposed numbering system. For instance, S341 lies at position 0′ in TM6, and T1134 lies at position +7′ in TM12. The region of highest discrimination between anions would consist of amino acid residues between 0′ and +3′. (C) Molecular models of TM6 and TM12. Amino acid residues are shown in ribbon form for the full predicted length of each TM domain (from +11′ to −12′). Stippled areas represent the side chains from amino acids at positions +7′, +3′, 0′ and −4′, which may contribute to binding sites for anions and open-channel blockers.

envision a structure similar to that shown in Fig. 6A, which proposes a pore lined by four transmembrane domains (TM5, TM6, TM11 and TM12). A design similar to this has been proposed for ligand-gated channels (Lester, 1992), which also line their pores with α-helices rather than a mixture of α-helices and β-strands as in the KcsA K^+ channel (Doyle et al., 1998). The α-helices in our fantasy model are oriented such that they are tilted and do not lie perpendicular to the membrane surface (there is no *a priori* reason to assume that TM helices are inserted at right angles to the membrane surface). Further, the extracellular ends of these domains are tilted towards each other, such that the cytoplasmic vestibule is larger than the outer vestibule. By deflecting the tilt tangentially to the axis of the pore, the helices would most closely approach each other at some distance from the extracellular end, rather than forming a point at the extracellular end. This structure, similar to the poles that make up a teepee, would result in a region of close apposition. This model is consistent with studies of the determinants of selectivity and open-channel block. The large inner vestibule is predicted from our studies showing that glibenclamide and DPC can occupy the pore at the same time. The small outer vestibule is predicted from the inability of blockers to reach their binding sites from the outside and from the short length

predicted for the loops between TM domains 5–6 and 11–12. The region of close apposition, where the poles of the teepee cross, may form the region of high discrimination, where selectivity is greatest, and may also be the site where open-channel blockers plug the pore. It is unlikely that the narrowest region lies towards the cytoplasmic end, as recently proposed (Akabas, 2000), given the voltage-dependence and distinct dependence upon side of application of block by DPC, NPPB, glibenclamide and gluconate. However, the multi-ion character of the CFTR pore suggests the presence of multiple binding sites. It is unlikely that all the binding sites would lie in this narrow region – others may lie towards the cytoplasmic end of the pore.

One prediction from the model is that the pore-lining residues from any TM domain contributing to the pore may not all fall on one face of the TM domain. Note that, even though the α-helices are not perpendicular to the membrane, the pore itself still is. Therefore, we would predict that the active surface of these TM domains would not form a line on a helical net parallel to the axis of the helix. Rather, the contributing region of these helical nets should be tilted off the axis of the α-helix, so that it can be parallel to the pore. Fig. 5 shows that this may be the case for TM6. Further, on each α-helix would be stretches where permeation is highly sensitive to mutation and stretches that are less sensitive to mutation. We propose that S341 (at 0′) and T338 (at +3′) contribute to the region of close apposition, because mutations in this region affect many properties of permeation (McCarty and Zhang, 1999). Indeed, these hydroxylated residues may play the largest role in conferring selectivity in CFTR, as suggested for ligand-gated ion channels (see Lester, 1992). Amino acid residues more extracellular to the +3′ position may contribute to the outer vestibule, because mutations here do not affect anion selectivity greatly. This model allows the formation of testable hypotheses.

Conclusions and perspective

The map of the permeation pathway in CFTR is far from complete. Our working hypothesis is that the pore in this channel consists of surfaces provided by transmembrane helices 5, 6, 11 and 12. With this preliminary three-dimensional picture in hand, in-depth structure/function studies are under way to formalize the positioning of residues within the volume of the pore and to identify the roles that these residues serve in constructing the energy profile of the pore. We hope that a detailed understanding of how CFTR works in normal cells may lead to the development of therapeutic strategies to enhance the function of mutant channels in cells of cystic fibrosis patients. This information may facilitate the rational design of therapeutic molecules for activation of CFTR channels in cystic fibrosis patients and inhibition of CFTR channels for treatment of secretory diarrhea and polycystic kidney disease.

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