Rectification of Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel Mediated by Extracellular Divalent Cations

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ABSTRACT We report here distinct rectification of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel reconstituted in lipid bilayer membranes. Under the symmetrical ionic condition of 200 mM KCl (with 1 mM $MgCl_2$ in *cis* intracellular and 0 $MgCl_2$ in *trans* extracellular solutions, pH in both solutions buffered at 7.4 with 10 mM HEPES), the inward currents (intracellular → extracellular chloride movement) through a single CFTR channel were ~20% larger than the outward currents. This inward rectification of the CFTR channel was mediated by extracellular divalent cations, as the linear current-voltage relationship of the channel could be restored through the addition of millimolar concentrations of $MgCl_2$ or $CaCl_2$ to the *trans* solution. The dose responses for $[Mg]_o$ and $[Ca]_o$ had half-dissociation constants of 152 ± 72 μM and 172 ± 40 μM, respectively. Changing the pH buffer from HEPES to *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid did not alter rectification of the CFTR channel. The nonlinear conductance property of the CFTR channel seemed to be due to negative surface charges on the CFTR protein, because in pure neutral phospholipid bilayers, clear rectification of the channel was also observed when the extracellular solution did not contain divalent cations. The CFTR protein contains clusters of negatively charged amino acids on several extracellular loops joining the transmembrane segments, which could constitute the putative binding sites for Ca and Mg.

INTRODUCTION

Cystic fibrosis is a lethal genetic disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (Quinton, 1990; Welsh and Smith, 1993; Tsui, 1995). CFTR is a chloride channel, the opening of which requires cAMP-dependent protein kinase (PKA) phosphorylation and intracellular ATP binding and hydrolysis (Gadsby et al., 1995; Frizzell, 1995). The predicted topology of CFTR contains two membrane-spanning domains that form part of the chloride conduction pore, and three cytoplasmic domains that regulate the channel activity: two nucleotide-binding domains consisting of the ATP-binding sites, and a regulatory domain containing multiple serine residues that can be phosphorylated by PKA (Riordan et al., 1989).

One of the identifying characteristics of the CFTR channel is its linear current-voltage relationship. Previous patch-clamp studies (Tabcharani et al., 1991; Egan et al., 1992; Carson and Welsh, 1995) and bilayer reconstitution measurements (Bear et al., 1992; Tilly et al., 1992; Gunderson and Kopito, 1994; Xie et al., 1995; Tao et al., 1996a; Ma et al., 1996) with single CFTR channels identified a linear conductance chloride channel of 7–13 pS. The different conductance values probably reflect the different ionic conditions in which the single-channel currents were measured, and to some extent the different compositions of membrane phospholipids in different tissues. Our recent studies show that chloride transport through CFTR is sensitive to surface charges on the bilayer membrane, suggesting that the conduction pore of the CFTR channel resides within the lipid bilayer (Tao et al., 1996b).

In the present studies we examined the current-voltage relationship of the single CFTR channel reconstituted in lipid bilayer membranes. Recording under symmetrical solutions of KCl, we observed a significant inward rectification of the CFTR channel that is mediated by divalent cations in the extracellular solution. Our data provide evidence that CFTR carries negative charges located on the extracellular side of the chloride channel.

MATERIALS AND METHODS

Heterologous expression of CFTR in HEK 293 cells

The wild-type CFTR cDNA (Drumm et al., 1990) was subcloned into the eukaryotic expression vector pCEP4 between the *Nhe*I and *Xho*I restriction sites. A human embryonic kidney cell line (293-EBNA HEK; Invitrogen) was used for transfection and expression of the wild-type CFTR protein. The cell line contains a vector pCMV-EBNA, which constitutively expresses the Epstein-Barr virus (EBV) EBNA-1 gene product, thus allowing immediate replication of the transfected pCEP4 vector. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% glutamine. Geneticin (G418, 250 μg/ml) was added to cell culture media for the continuous selection of cells containing PCMV-EBNA vector. The parent cell line was grown to confluence in a 37°C incubator with 5% CO_2 and passed 1:5 2 days before the gene transfer. pCEP4(CFTR) was then introduced into the cells with the lipofectin reagent. Two days after transfection, the cells were passed and selected for hygromycin resistance in a medium containing hygromycin B at 260 μg/ml. After 3–4 weeks in selection, the cells were harvested and used for the isolation of microsomal vesicles and reconstitution studies in

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lipid bilayers. The expression of CFTR protein was verified by Western blot using antibody against the R domain of CFTR for each transfection (Xie et al., 1995).

The HEK 293 cells transfected with pCEP4(CFTR) could maintain high levels expression of CFTR proteins for a period of 5–7 weeks; after that the amount of CFTR proteins started to decline. Because of this down-regulation of CFTR expression during the stable selection process, transient expression without selection for hygromycin resistance was used in the later stage of this study. 48–72 h after transfection of pCEP4(CFTR) into the HEK 293 cells with the lipofectamine reagent, the HEK 293 cells were harvested, from which microsomal membrane vesicles were isolated. These vesicles, containing sufficient amounts of CFTR proteins detected with a Western blot, were used in the bilayer reconstitution studies.

Isolation of microsomal membrane vesicles from HEK 293 cells

The HEK 293 cells transfected with pCEP4(CFTR) were harvested and homogenized using a combination of hypotonic lysis and Dounce homogenization, in the presence of protease inhibitors (5 μM diisopropyl fluorophosphate, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 10 mg/ml benzamidine) (Xie et al., 1995; Tao et al., 1996a). The microsomal membrane vesicles were isolated after sequential centrifugation at 600 $\times$ g and 100,000 $\times$ g. The vesicles were resuspended in a prephosphorylation buffer (250 mM sucrose, 5 mM Mg-ATP, 100 units/ml PKA catalytic subunit, 10 mM HEPES-Tris, pH 7.2) at a protein concentration of 3–7 mg/ml, and stored at −75°C until use. Usually 1–3 μl of microsomal membrane vesicles was added to the *cis* solution for the reconstitution of CFTR channels in the lipid bilayer membranes.

Reconstitution of CFTR channels in lipid bilayer membranes

Lipid bilayer membranes were formed across an aperture of ~200 μm diameter with a lipid mixture of phosphatidylethanolamine/phosphatidylserine/cholesterol (6:6:1). The lipids were dissolved in decane at a concentration of 40 mg/ml (Tao et al., 1996a). The recording solutions contained: *cis* (intracellular, 1-ml volume), 200 mM KCl, 2 mM ATP, 1 mM $MgCl_2$, and 10 mM HEPES-Tris (pH 7.4); *trans* (extracellular, 3 ml volume), 50 mM KCl, 10 mM HEPES-Tris (pH 7.4). PKA catalytic subunit (Promega) (50 units/ml) was always present in the *cis* solution. Because the agonists for CFTR channel (ATP and PKA) were only present in the *cis* solution, this condition selected only CFTR channels that were oriented in the bilayer membrane in the *cis* intracellular, *trans* extracellular manner.

After incorporation of a single CFTR channel into the bilayer membrane, KCl in the *trans* solution was adjusted to 200 mM (with the addition of 161 μl of 3 M KCl stock solution), to allow measurements of currents under symmetrical KCl condition. The *trans* solution was mixed well before measurements of single-channel currents were performed.

The data presented in this study were obtained with six different preparations of microsomal membrane vesicles isolated from the HEK 293 cells transfected with wild-type CFTR cDNA (three from stable expression and three from transient expression). The total number of experiments was 240. In terms of single-channel conductance and gating kinetics of the CFTR channel, no significant difference was observed with the CFTR proteins stably or transiently expressed in HEK 293 cells.

Single-channel recording and data analyses

To maintain stability of the bilayer membrane and the CFTR channel activities, the bilayer membrane was kept at a holding potential of 0 mV, from which different pulse protocols were applied to measure currents through the single CFTR channels: 1) step changes to positive and negative voltages of equal amplitude (see Fig. 1) to measure single-channel currents through CFTR, and to compare currents at positive and negative voltages; and 2) the incremental increase of voltage from −100 to +100 mV (ramp pulse protocol; see Fig. 2) to measure the instantaneous current-voltage relationship of the CFTR channel. Each test pulse had a duration of either 2 or 5 s, and the interval between consecutive pulses was 10 s.

Single-channel currents were recorded with an Axopatch 200A patch-clamp unit (Axon Instrument). Data acquisition and pulse generation were performed with a 486 computer and 1200 Digidata A/D-D/A convertor (Axon Instrument). The currents were sampled at 1–2.5 ms/point and filtered at 100 Hz through an 8-pole Bessel filter. Data analyses were performed with pClamp, TIPS, and custom softwares. The analysis of single-channel data started with the construction of controls, by averaging the infrequent "null" sweeps. The controls, which contained only the capacitative transients, were subtracted from the total currents to obtain the "test minus control" currents (Ma et al., 1991). All subsequent analyses were performed with these tests minus controls, the single-channel currents without capacitative transients.

Our previous studies identified three conductance states associated with the single CFTR channel: the 8-pS full open state, and sub-open states of 6 pS and 2.7 pS (Tao et al., 1996a). To focus on the full open state of the CFTR channel, and to obtain reliable estimates of the current amplitude at

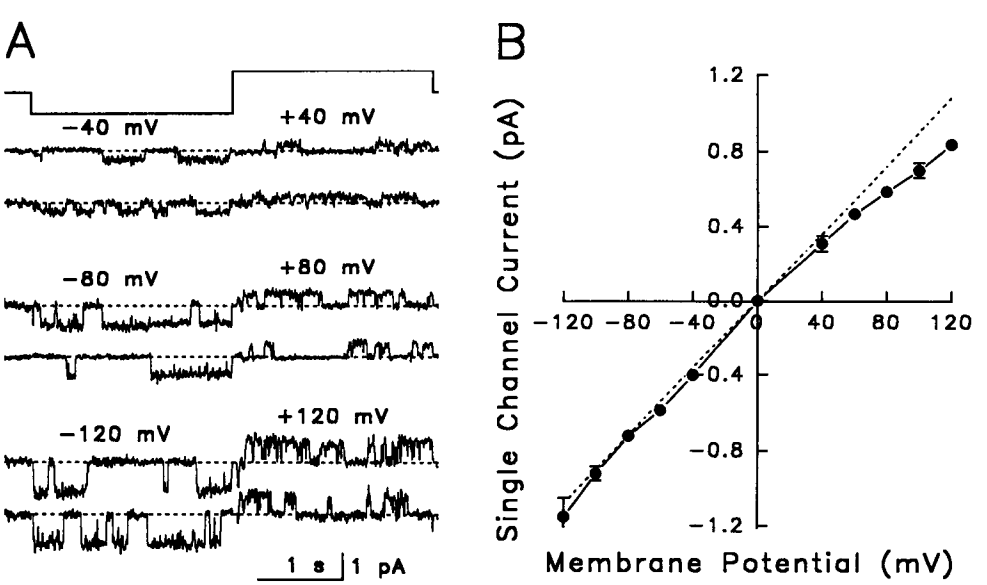

FIGURE 1 Inward rectification of CFTR chloride channel in symmetrical KCl solution. The selected single-channel traces at the given test potentials were taken from the same bilayer experiment, with the pulse protocol shown at the top (*A*). The data points are averages of seven bilayer experiments (mean ± SD) (*B*). Notice an inward rectification of the *I-V* relationship. The dotted line represents a linear conductance of 8.2 pS. The recording solution contained: *cis*, 200 mM KCl, 2 mM ATP, 1 mM $MgCl_2$, 50 units/ml C-subunit of PKA, and 10 mM HEPES-Tris (pH 7.4); *trans*, 200 mM KCl, 10 mM HEPES-Tris (pH 7.4).

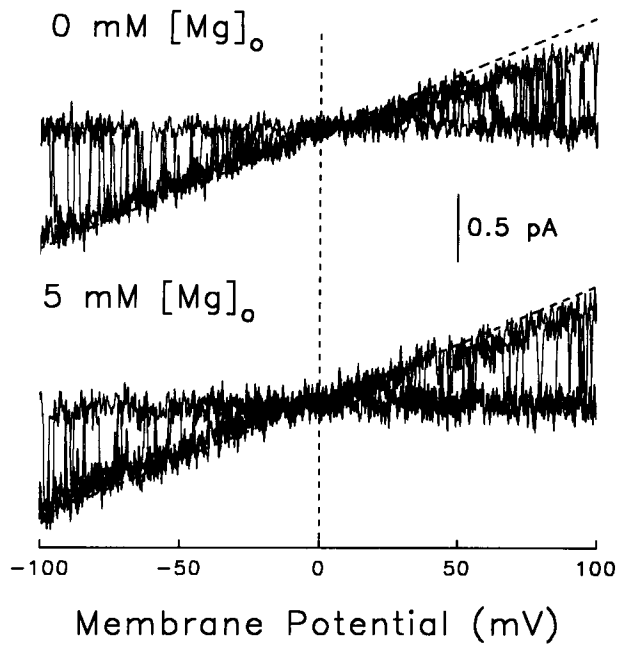

FIGURE 2 Instantaneous current-voltage relationship of the CFTR channel. A ramp pulse protocol was applied to a single CFTR channel incorporated into the bilayer membrane. The pulse ran from −100 mV to +100 mV over a duration of 5 s. The instantaneous current-voltage relationships of the CFTR channel were represented by six consecutive episodes overlaid on top of each other, in the absence of extracellular Mg (*top*), and after the addition of 5 mM $MgCl_2$ to the extracellular solution (*bottom*). The current carrier was 200 mM symmetrical KCl.

a given test potential, traces were selected that did not exhibit transition to the subconductance states. The amplitude histogram was fitted with a Gaussian distribution function, to have an estimate of open-channel current from individual experiments. The estimates from multiple experiments (usually $n > 6$) were averaged to obtain the mean current amplitude (with its standard deviation) at the given test potential and ionic condition.

RESULTS

Inward rectification of the CFTR channel

The HEK 293 cells transfected with pCEP4 vector containing the wild-type CFTR cDNA gave high-level expression of the mature 170-kDa CFTR proteins, detected with a Western blot using antibody against the R domain of CFTR (Xie et al., 1995; Ma et al., 1996). With the microsomal membrane vesicles isolated from these cells, activities of single CFTR channels could be routinely measured in the lipid bilayer membrane. Opening of the channel strictly required the presence of ATP and PKA (catalytic subunit) in the *cis* intracellular solution, and the channel activity was sensitive to blockade by diphenylcarboxylate (DPC).

To measure the current-voltage (*I-V*) relationship of the CFTR channel, we used the following pulse protocol: the bilayer was held at 0 mV and pulsed to negative followed by positive voltages of equal amplitude (Fig. 1 *A*). Under the symmetrical ionic condition of 200 mM KCl (with 1 mM $MgCl_2$ in *cis* intracellular and 0 $MgCl_2$ in *trans* extracellular solutions, pH in both solutions buffered at 7.4 with 10 mM HEPES), the inward currents (*cis* → *trans* Cl^- movement) through a single CFTR channel were consistently larger than the outward currents (Fig. 1 *A*). For example, the full open state of the CFTR channel had a current amplitude of −0.67 ± 0.12 pA at −80 mV, which is ~20% larger than that at +80 mV (I_{+80} = +0.54 ± 0.11 pA). The complete *I-V* curve exhibited a small but notable inward rectification, in a voltage range from −120 to +120 mV (Fig. 1 *B*).

This distinct rectification of the CFTR channel was not observed in previous patch-clamp measurements of CFTR channels (Tabcharani et al., 1991; Egan et al., 1992; Carson and Welsh, 1995), nor was it obvious in other bilayer reconstitution studies of CFTR channels (Bear et al., 1992; Gunderson and Kopito, 1994). The main difference between our experiments and the others was in the composition of the recording solutions. In this study we used a *trans* extracellular solution that did not contain any divalent cations, unlike previous studies, which always included millimolar concentrations of Ca or Mg in the extracellular solution (2 mM Ca, Tabcharani et al., 1991; 2 mM Ca, Egan et al., 1992; 5 mM Ca and 2 mM Mg, Carson and Welsh, 1995; 1 mM Mg and 2 mM Ca, Bear et al., 1992; 5 mM Mg, Gunderson and Kopito, 1994). We tested whether the inward rectification of the CFTR channel we observed was due to the absence of divalent cations in the extracellular solution.

Effects of extracellular Mg on CFTR channel

We measured the instantaneous current-voltage relationship of the channel, using a ramp pulse protocol (−100 → +100 mV, 0.04 mV/ms) (Fig. 2). With no Mg present in the extracellular solution, the movement of chloride ions through the CFTR channel was asymmetrical. In particular, the outward chloride currents deviated significantly from the linear *I-V* relationship (Fig. 2, *top*). Thus, similar to the stationary *I-V* curve (Fig. 1), the instantaneous *I-V* relationship of the CFTR channel also displayed significant inward rectification.

When 5 mM $MgCl_2$ was added to the extracellular solution, the outward chloride currents increased, and the *I-V* curve became linear (Fig. 2, *bottom*) in multiple experiments ($n > 40$). The dotted lines in Fig. 2 represent a linear *I-V* curve with a slope conductance of 8.5 pS.

The amplitude histograms shown in Fig. 3 were constructed from consecutive single-channel episodes obtained with pulse protocols similar to those shown in Fig. 1. As can be seen, when no Mg was present in the extracellular solution, the outward currents (+80 mV) were significantly smaller than the inward currents (−80 mV) (Fig. 3 *a*). Subsequent addition of $MgCl_2$ to the extracellular solution led to a significant increase in the outward currents, without affecting the amplitude of the inward currents. In the presence of 5 mM Mg, the outward currents were approximately equal to the inward currents (Fig. 3, *d* and *e*). The large

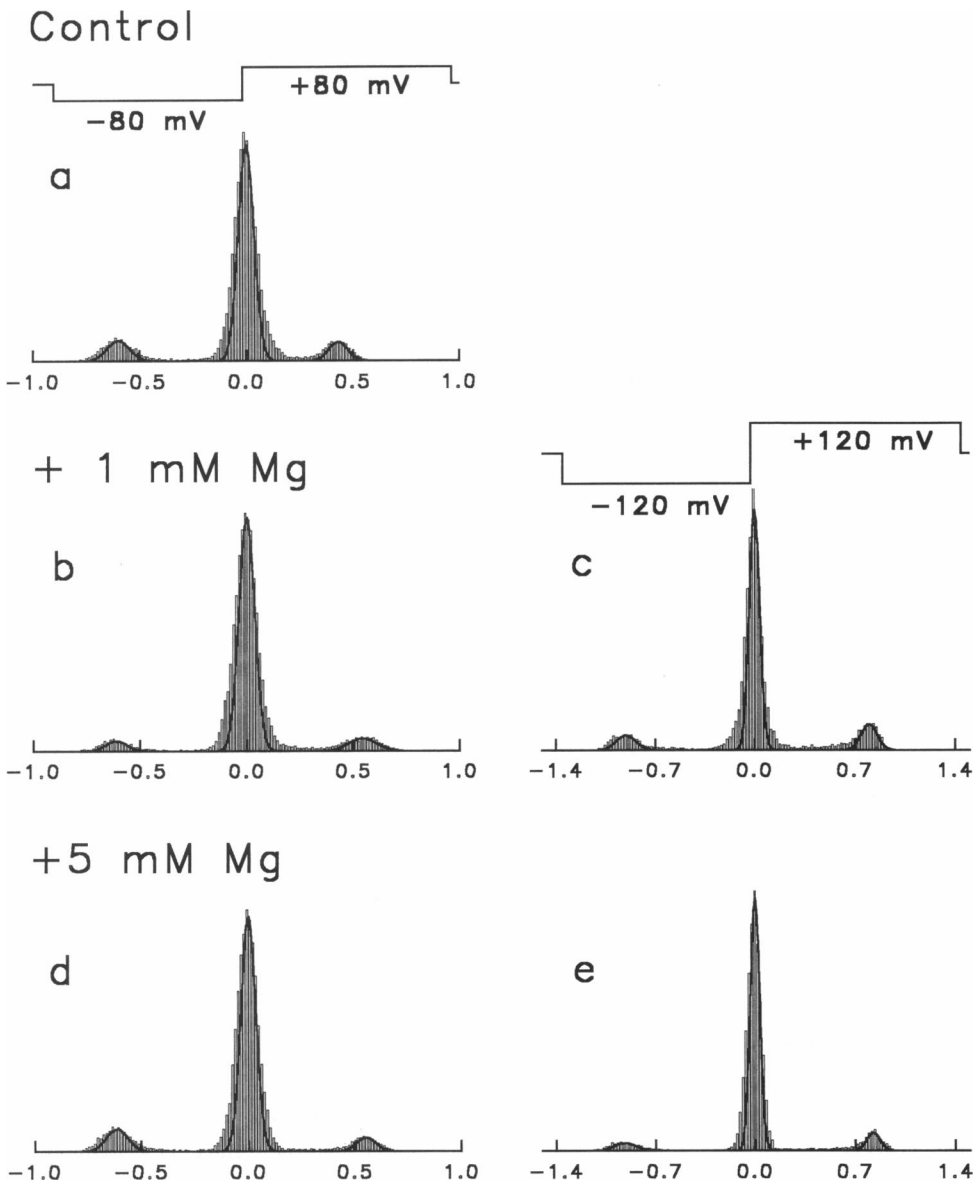

FIGURE 3 Current amplitude histogram of the CFTR channel. The all point amplitude histograms were obtained from a continuous experiment. Each histogram was generated with 16 consecutive episodes of single-channel currents obtained with a similar pulse protocol given in Fig. 1 *A*. The traces were digitally filtered at 40 Hz. The left panels represent test pulses to −80 mV and +80 mV (*a, b, d*), and the right panels represent test pulses to −120 mV and +120 mV (*c, e*). The solid lines represent the best fit with Gaussian distribution functions. The best-fit parameters are given below (mean ± SD). (*a*) Control, on Mg added to the *trans* solution: I_{-80} = −0.613 ± 0.075 pA, I_0 = 0 ± 0.052 pA, I_{+80} = 0.427 ± 0.063 pA. (*b* and *c*) +1 mM Mg, I_{-80} = −0.611 ± 0.071 pA, I_0 = 0 ± 0.052 pA, I_{+80} = 0.541 ± 0.095; I_{-120} = −0.905 ± 0.099 pA, I_0 = 0 ± 0.052 pA, I_{+120} = 0.780 ± 0.079 pA. (*d* and *e*) +5 mM Mg, I_{-80} = −0.606 ± 0.072 pA, I_0 = 0 ± 0.049 pA, and I_{+80} = 0.560 ± 0.064; I_{-120} = −0.919 ± 0.109 pA, I_0 = 0 ± 0.047 pA, I_{+120} = 0.826 ± 0.067 pA.

standard deviations of the mean open current amplitudes (SD = 0.06–0.11) with the individual experiments were of two origins, the background noise (at 0 mV, SD = 0.052 pA) and the contribution of subconductance states. The difference in the mean current amplitudes at +80 mV and −80 mV was consistently seen in all of the experiments when Mg was not present in the extracellular solution ($n >$ 200).

The changes in the *I-V* relationship with the addition of $[Mg]_o$ (i.e., differences in single-channel currents at positive voltages) were significant, as shown in the titration experiment with extracellular Mg (Fig. 4). The difference in single-channel currents at −80 mV and +80 mV, expressed as the ratio of $(I_{-80} - I_{+80})/I_{-80}$, diminished as the concentration of extracellular Mg was increased from 1 μM to 2 mM, reaching zero at concentrations above 5 mM $[Mg]_o$

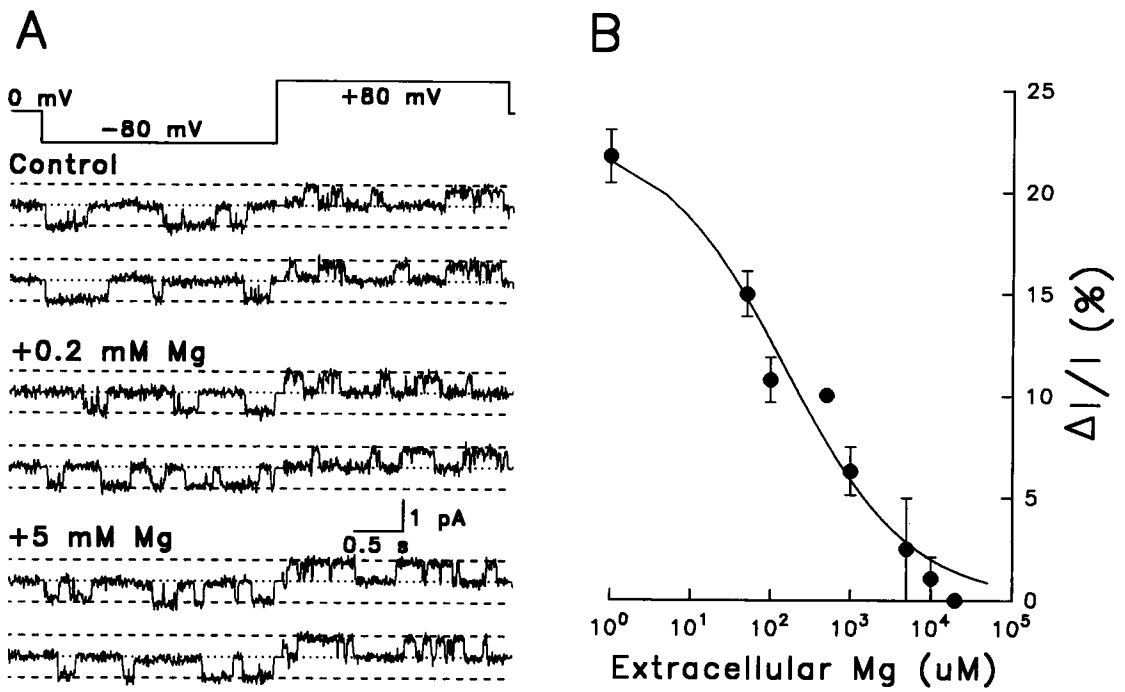

FIGURE 4 Mg-mediated rectification of CFTR chloride channel. (*A*) Selected single-channel currents at −80 and +80 mV were taken from the same bilayer experiment, with no extracellular $MgCl_2$ (control), after the addition of $MgCl_2$ to the extracellular solution (+0.2 mM Mg, +5 mM Mg). (*B*) The changes in single-channel currents, represented by $(I_{-80} - I_{+80})/I_{-80}$, were plotted as a function of the concentration of extracellular $[Mg]_o$. The solid line represents the best fit Hill equation: $Y = y_{max}/[1 + (X/K_d)^n]$, where $y_{max} = 21.8 \pm 2.3\%$, $K_d = 152 \pm 72$ μM, and $n = 0.66 \pm 0.11$.

(Fig. 4 *B*). The dose responses for $[Mg]_o$ had a dissociation constant (K_d) = 152 ± 72 μM, with a Hill coefficient (n_H) = 0.66 ± 0.11 (Fig. 4).

Rectification of CFTR channel mediated by extracellular divalent cations

To test whether the observed effects were specific for Mg, we substituted $CaCl_2$ for $MgCl_2$ in the extracellular solution. Ca was as effective as Mg in restoring the linear *I-V* relationship of the CFTR channel (Fig. 5). In addition, the half-dissociation constant for Ca was 172 ± 40 μM, similar to that for Mg. Thus charge interaction seems to be the main determinant in mediating rectification of the CFTR channel.

Because 2 mM ATP (Tris salt) was constantly present in the *cis* solution, and because ATP has been shown to permeate the CFTR channel (Reisin et al., 1994; Schweibert et al., 1995; but see also Grygorczyk et al., 1996), it is possible that the unidirectional movement of ATP through the CFTR channel could contribute to some degree of rectification in the CFTR channel. To test this hypothesis, 2 mM ATP was added to the extracellular solution. The addition of ATP did not result in significant changes in the single-channel currents at either −80 mV or +80 mV. The average single-channel currents were $I_{-80} = -0.65 \pm 0.13$ and $I_{+80} = +0.56 \pm 0.09$ at 2 mM $[ATP]_o$, which were not significantly different from the controls (Fig. 1). Thus the asymmetrical distribution of ATP in the recording solution did not contribute to the rectification of the CFTR channel.

Hanrahan and Tabcharani (1990) showed that different buffers had inhibitory effects on the outwardly rectifying chloride channel (ORCC). HEPES at a concentration of 10 mM could cause the inhibition of up to 25% of chloride current through ORCC, whereas *N*-tris-(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES), a related buffer, did not exert appreciable inhibition of ORCC (Hanrahan and Tabcharani, 1990). To test whether HEPES participates in the rectification of the CFTR channel, we substituted TES for HEPES in both *cis* and *trans* solutions. In 10 mM TES (pH titrated to 7.4 with Tris), the CFTR channel still exhibited clear rectification (Fig. 6). Moreover, the half-dissociation constant for Mg was 186 ± 50 μM in 10 mM TES, not significantly different from that in 10 mM HEPES (Fig. 4). Thus the buffer did not affect the rectification of the CFTR channel.

Taken together, the observed rectification of the CFTR channel was likely due to specific binding of divalent cations to the extracellular side of the channel.

Surface charge and CFTR channel

There are two possible mechanisms through which extracellular divalent cations could increase the outward chloride currents through the CFTR channel: neutralization of negative charges on the extracellular side of the CFTR protein, and screening of surface charges on the bilayer membrane.

To eliminate the contribution of membrane surface charges, we changed the composition of the lipid bilayer

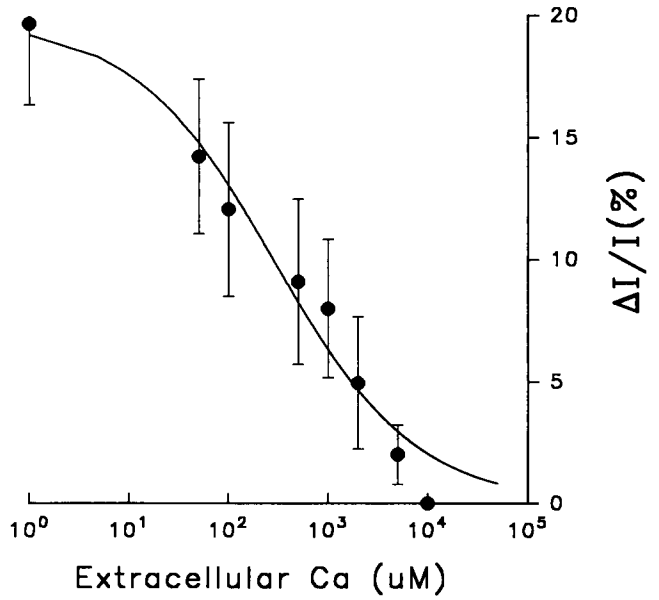

FIGURE 5 Effects of extracellular Ca on CFTR channel. Single-channel currents at −80 mV (I_{-80}) and +80 mV (I_{+80}) were measured in 200 mM symmetrical KCl, in the presence of 1 mM $MgCl_2$ (*cis*) and various concentrations of $CaCl_2$ added to the *trans* solution. The titration curve of $(I_{-80} - I_{+80})/I_{-80}$ versus $[Ca]_o$ had a y_{max} = 19.8 ± 1.8%, K_d = 172 ± 40 μM, and n = 0.65 ± 0.12.

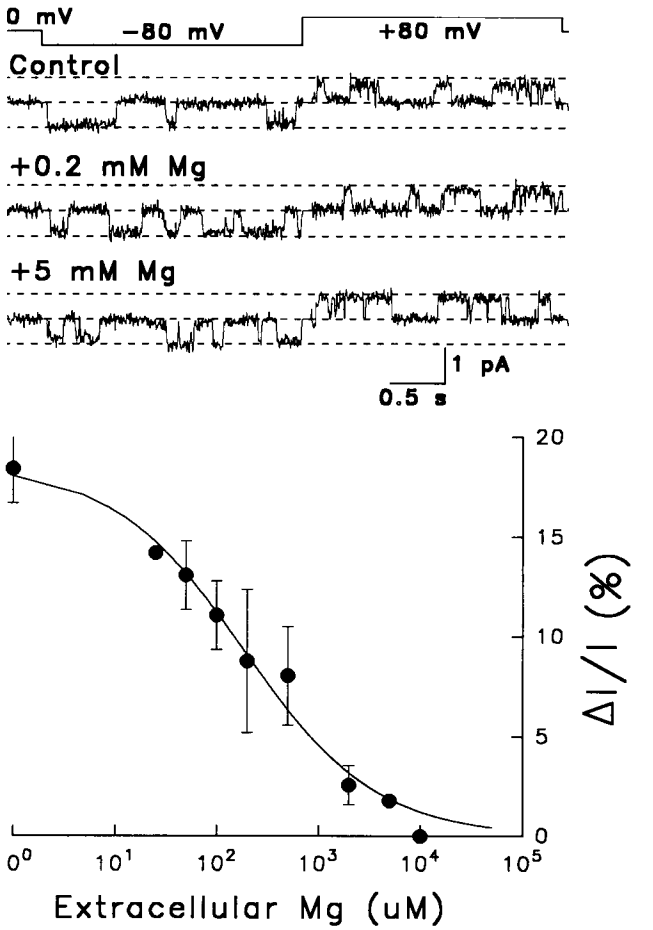

FIGURE 6 Measurement of the CFTR channel in TES buffer. The recording solution was the same as in Fig. 1, except that 10 mM TES was the pH buffer. Changing the pH buffer from HEPES to TES did not affect the single-channel currents at either −80 mV or +80 mV. The dose-response relationship for extracellular Mg ($\Delta I/I$ versus $[Mg]_o$) had a y_{max} = 18.7 ± 1.2%, K_d = 186 ± 50 μM, and n = 0.67 ± 0.09.

from PE:PS:cholesterol = 6:6:1 to 12:0:1 (Fig. 7). With the neutral phospholipid bilayer membrane (PE:PS = 12:0), both inward and outward chloride currents through the CFTR channel were significantly larger than those with the negatively charged bilayer membrane (PE:PS = 6:6). In PE:PS = 12:0, the measured single-channel currents at −80 and +80 mV were −0.86 ± 0.07 pA and 0.68 ± 0.09 pA in 200 mM symmetrical KCl, respectively. This showed that chloride movements through the CFTR channel (both inward and outward currents) were sensitive to surface charges on the bilayer membrane. A more detailed study of the modulation of CFTR channel by membrane surface charge has been presented in an abstract form (Tao et al., 1996b). However, the instantaneous *I-V* relationship of the CFTR channel still displayed significant inward rectification when the extracellular solution did not contain divalent cations (Fig. 7). The slope conductance at the negative voltage range was 11.3 ± 0.5 pS (represented by the dotted line in Fig. 7), and that in the positive voltage range was approximately 8 pS.

To further test the contribution of ionic strength to the rectification property of the CFTR channel, we did a series of single-channel measurements at 50 mM symmetrical KCl, with lipid bilayers made of PE:PS:cholesterol = 6:6:1 (Fig. 8 *A*) and 12:0:1 (Fig. 8 *B*). Two things can be noticed in Fig. 8. First, the current amplitudes (at both −80 mV and +80 mV) were larger in neutral lipid bilayer than in negatively charged lipid bilayer (compare Fig. 8 *B* with Fig. 8 *A*); this is consistent with the observation that chloride conduction through the CFTR channel is sensitive to membrane surface charges (see Fig. 7). Second, the difference in single-channel currents at −80 mV and +80 mV was larger in 50 mM KCl than that in 200 mM KCl (compare Fig. 8 with Fig. 1). Such stronger rectification of the CFTR channel at lower ionic concentrations further suggests an asymmetrical distribution of surface charges near the intracellular and extracellular portions of the CFTR channel.

The fact that the CFTR channel maintained intrinsic rectification even in the absence of surface charges on the bilayer membrane suggests a direct interaction of divalent cations with the CFTR molecule, most likely mediated by negative surface charges on the extracellular portion of the CFTR channel.

DISCUSSION

An open ion channel is a pore that selectively translocates ions from one side of the membrane to the other. Within the voltage range from −100 to +100 mV, the channel proteins

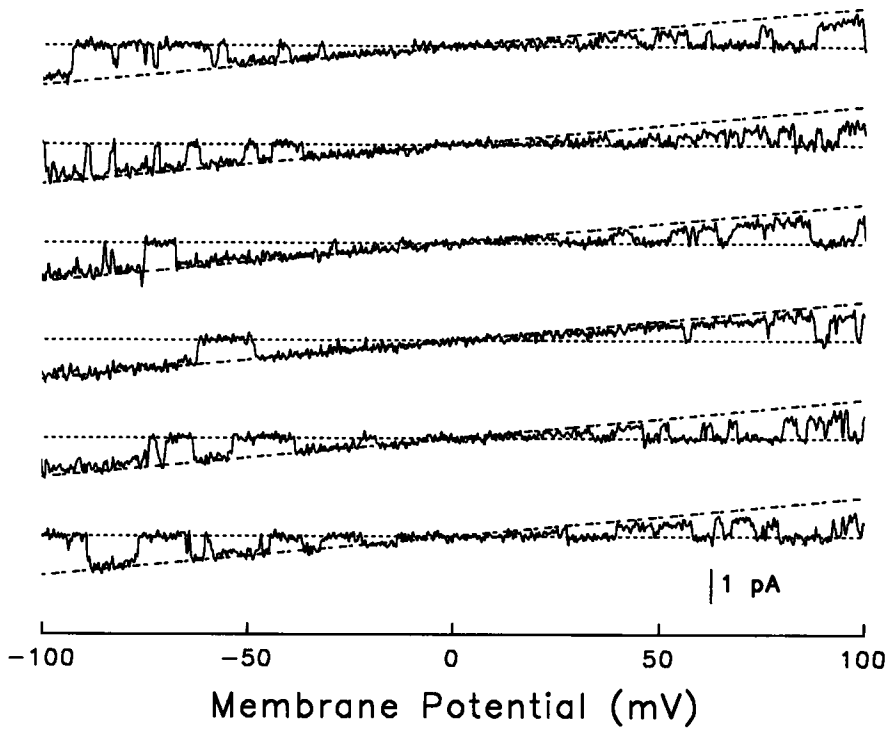

FIGURE 7 Rectification of the CFTR channel in neutral bilayer membranes. A single CFTR channel was incorporated into a lipid bilayer membrane made with a lipid composition of PE:PS:cholesterol = 12:0:1 (all previous experiments were with PE:PS:cholesterol = 6:6:1). The records shown were consecutive episodes, obtained with a ramp pulse protocol (see Fig. 2). The dotted lines correspond to a slope conductance of 11.3 pS. Notice that the outward currents are smaller than the inward currents.

usually behave like an ohmic conductor, i.e., the amount of ions moved through the channel, independent of the direction the ions move (into or out of the cells), is proportional to the amount of driving force applied across the membrane (Hille, 1992). A channel that exhibits a nonlinear current-voltage relationship (under symmetrical ionic conditions) can be the result of several possible factors:

1. Perturbation of the local concentration of the permeant ions near the conduction pore of the channel due to asymmetrical distribution of surface charges on the channel protein or on the bilayer membrane. For example, the ryanodine receptor/calcium release channel located in the sarcoplasmic reticulum membrane of striated muscles contains negative charges on the luminal side of the channel

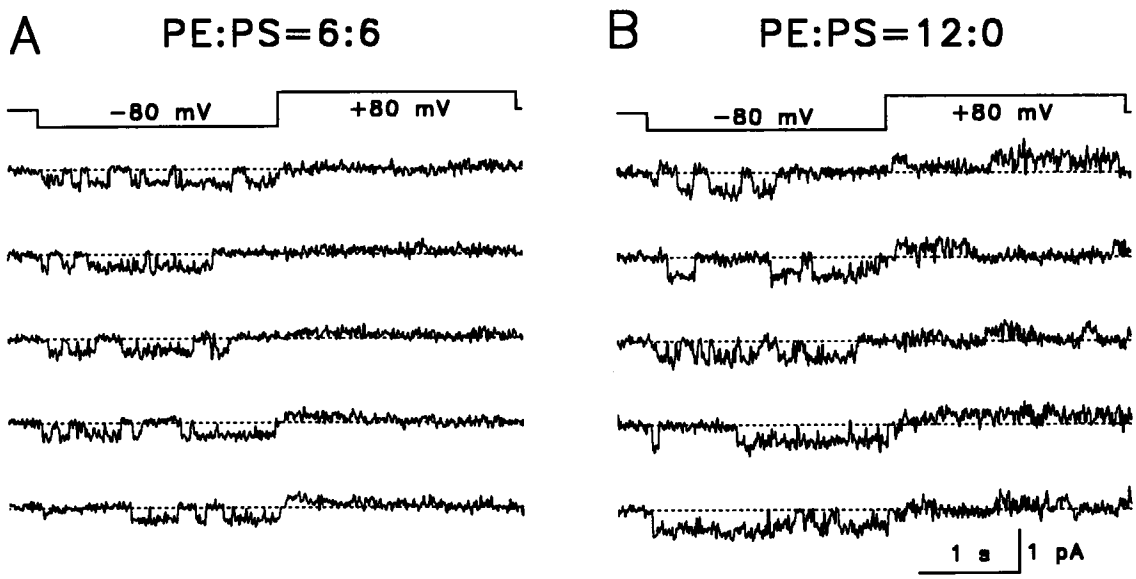

FIGURE 8 Rectification of CFTR channel in 50 mM symmetrical KCl solution. The recording solution was the same as in Fig. 1, except that 50 mM symmetrical KCl was the current carrier. The single-channel traces shown in *A* were obtained with a CFTR channel incorporated into a lipid bilayer membrane formed with PE:PS:cholesterol = 6:6:1, and those in *B* were obtained from a CFTR channel incorporated into a lipid bilayer membrane formed with PE:PS:cholesterol = 12:0:1.

pore (Takeshima et al., 1989), which facilitates the movement of calcium ions from inside the sarcoplasmic reticulum to the myoplasmic space, thus leading to the asymmetrical feature of the current-voltage relationship (particularly at low ionic concentrations) (Tu et al., 1994).

2. Voltage-dependent block of the open channel through competition of nonpermeant ionic species or ligands with the permeant ions. It has been well demonstrated that magnesium ions or endogenous polycations bind to the intracellular portion of the inward rectifier potassium channel, which leads to a voltage-dependent block of the outward potassium currents (Ficker et al., 1994; Lopatin et al., 1994).

The present study showed that a single CFTR channel exhibited intrinsic inward rectification, i.e., the channel allows more chloride ions moving out of the cell, when the extracellular solution did not contain divalent cations. This rectification was not observed in previous studies, including patch-clamp and bilayer reconstitution of single CFTR channels, probably because the previous studies were always conducted with calcium or magnesium present in the extracellular solution. Divalent cations are necessary to form tight seals between the microelectrode and the membrane patch; thus it is essential to have millimolar concentrations of $CaCl_2$ or $MgCl_2$ in both intracellular and extracellular solutions when patch-clamp studies are performed with the CFTR channel (Tabcharani et al., 1991; Egan et al., 1992; Carson and Welsh, 1995).

The observed rectification of the CFTR channel in the bilayer membrane is independent of the concentration of ATP in the extracellular solution or the buffer composition (HEPES or TES) used in the recording solution. This excludes a potential voltage-dependent blocking mechanism as the basis for the asymmetrical conduction property of the CFTR channel. In addition, although the negative surface charges on the bilayer membrane can modulate the chloride conductance through the CFTR channel (Tao et al., 1996b), it does not alter the rectification property of the CFTR channel (Fig. 6). Thus it is likely that an asymmetrical distribution of surface charge on the CFTR protein itself is responsible for the inward rectification of the chloride channel.

The increase in the outward chloride currents caused by the addition of extracellular Mg (or Ca) could be due to either specific binding of divalent cations to the CFTR protein or screening of negative charges near the extracellular pore region of the CFTR channel. Our present results, however, are insufficient to distinguish these mechanisms. To further test the effects of "binding" versus "screening," more experiments are necessary (such as measurements of the *I-V* relationship at different ionic concentrations and in the presence of different extracellular divalent cations). In addition, specific assumptions have to be made about the location of the binding site and the density of the surface charges. Because of the difficulty with the precise measurement of the difference in single-channel currents induced by the addition of extracellular divalent cations, we could not proceed with more quantitative analyses of the effects of divalent cations on the CFTR channel.

The putative 12 transmembrane (TM) segments of CFTR are joined by six extracellular and four intracellular loops of different peptide sequences. Our previous studies showed that the first and second intracellular loops, linking TM2 and TM3 and TM4 and TM5, respectively, played important roles in the function of the CFTR channel. The deletion of 19 amino acids from the second intracellular loop, Δ19 CFTR (Xie et al., 1995), and the deletion of 30 amino acids from the first intracellular loop, Δexon 5 CFTR (Xie et al., manuscript submitted for publication), caused a processing problem in CFTR, as well as altered the conductance states of the CFTR chloride channel. Of the six extracellular loops in the CFTR molecule, the first extracellular loop (linking TM1 and TM2) and the sixth extracellular loop (linking TM11 and TM12) contain negatively charged amino acids at residues D110, D112, E115, E116 (first loop), and E1124, E1126 (sixth loop). Anderson et al. (1991) and Akabas et al. (1994) showed that TM1 participates in the formation of the chloride channel conduction pore, as specific mutations within the TM1 region altered the anion selectivity of the chloride channel. McDonough et al. (1994) demonstrated that residues within the TM12 region are involved in the voltage-dependent block of the CFTR channel by extracellular diphenylcarboxylate (DPC). The studies of Tabcharani et al. (1993) showed that TM6 is also involved in the conduction process of the CFTR channel, as point mutations of K335E and R347D altered the multi-ion pore behavior of the CFTR channel. In addition, R117H, one of the common disease-causing mutations in cystic fibrosis, is located in the first extracellular loop of CFTR (Tsui, 1995). Based on these studies, it is possible that the first and sixth extracellular loops could reside close to, or even participate in the formation of, the chloride conduction pore of the CFTR channel. The negatively charged amino acid residues located on the extracellular loops of CFTR could form a putative binding site for calcium and magnesium, and thus contribute to the rectification of the CFTR channel. Further identification of the specific amino acid residues that participate in the rectification process of the CFTR channel should reveal important information about the topology and structure of the CFTR channel.

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REFERENCES

Akabas, M. H., C. Kaufmann, T. A. Cook, and P. Archdeacon. 1994. Amino acid residues lining the chloride channel of cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 269:14865–14868.

Anderson, M. P., R. J. Gregory, S. Thompson, D. W. Souza, S. Paul, R. C. Mulligan, A. E. Smith, and M. J. Welsh. 1991. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science.* 253:202–205.

Bear, C. E., C. Li, N. Kartner, R. J. Bridges, T. J. Jensen, M. Ramjeesingh, and J. R. Riordan. 1992. Purification and functional reconstitution of the

cystic fibrosis transmembrane conductance regulator (CFTR). *Cell.* 68: 809–818.

Carson, M. R., and M. J. Welsh. 1995. Structural and functional similarities between the nucleotide-binding domains of CFTR and GTP-binding proteins. *Biophys. J.* 69:2443–2448.

Drumm, M. L., H. A. Pope, W. H. Cliff, J. M. Rommens, S. A. Marvin, L.-C. Tsui, F. S. Collins, R. A. Frizzel, and J. M. Wilson. 1990. Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. *Cell.* 62:1227–1233.

Egan, M., T. Flotte, S. Afione, R. Solow, P. L. Zeitlin, B. J. Carter, and W. B. Guggino. 1992. Defective regulation of outwardly rectifying Cl^- channels by protein kinase A corrected by insertion of CFTR. *Nature.* 358:581–584.

Ficker, E., M. Taglialatela, B. A. Wible, C. M. Henley, and A. M. Brown. 1994. Spermine and spermidine as gating molecules for inward rectifier K channels. *Science.* 266:1068–1072.

Frizzell, R. A. 1995. Functions of the cystic fibrosis transmembrane conductance regulator protein. *Am. J. Respir. Crit. Care Med.* 151: S54–S58.

Gadsby, D. C., G. Nagel, and T. C. Hwang. 1995. The CFTR chloride channel of mammalian heart. *Annu. Rev. Physiol.* 57:387–416.

Grygorczyk, R., J. A. Tabcharani, and J. W. Hanrahan. 1996. ATP conductance of the CFTR chloride channel. *Biophys. J.* 70:A71.

Gunderson, K. L., and R. R. Kopito. 1994. Effects of pyrophosphate and nucleotide analogs suggest a role for ATP hydrolysis in cystic fibrosis transmembrane regulator channel gating. *J. Biol. Chem.* 269: 19349–19353.

Hanrahan, J. W., and J. A. Tabcharani. 1990. Inhibition of an outwardly rectifying anion channel by HEPES and related buffers. *J. Membr. Biol.* 116:65–77.

Hille, B. 1992. Ionic Channels of Excitable Membranes. 2nd Ed. Sinauer Associates, Sunderland, MA.

Lopatin, A. N., E. N. Makhina, and C. G. Nichols. 1994. Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature.* 372:366–369.

Ma, J., C. Mundina-Weilenmann, M. M. Hosey, and E. Rios. 1991. Dihydropyridine-sensitive skeletal muscle Ca channels in polarized planar bilayers. I. Kinetics and voltage dependence of gating. *Biophys. J.* 60:890–901.

Ma, J., J. Tasch, T. Tao, J. Y. Zhao, J. Xie, M. L. Drumm, and P. B. Davis. 1996. Phosphorylation dependent block of CFTR chloride channel by exogenous R domain protein. *J. Biol. Chem.* 271:7351–7356.

McDonough, S., N. Davidson, H. A. Lester, and N. A. McCarty. 1994. Novel pore-lining residues in CFTR that govern permeation and open-channel block. *Neuron.* 13:623–634.

Quinton, P. M. 1990. Cystic fibrosis: a disease of electrolyte transport. *FASEB J.* 4:2709–2717.

Reisin, I. L., A. G. Prat, E. H. Abraham, J. F. Amara, R. J. Gregory, D. A. Ausiello, and H. F. Cantiello. 1994. The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel. *J. Biol. Chem.* 269:20584–20591.

Riordan, J. R., J. M. Rommens, B. S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.-L. Chou, M. L. Drumm, M. C. Iannuzzi, F. S. Collins, and L.-C. Tsui. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science.* 245:1066–1073.

Schwiebert, E. M., M. E. Egan, T. H. Hwang, S. B. Fulmer, S. S. Allen, G. R. Cutting, and W. B. Guggino. 1995. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell.* 81:1063–1073.

Tabcharani, J. A., X. B. Chang, J. R. Riordan, and J. W. Hanrahan. 1991. Phosphorylation-regulated Cl channel in CHO cells stably expressing the cystic fibrosis gene. *Nature.* 352:628–631.

Tabcharani, J. A., J. M. Rommens, Y. X. Hou, X. B. Chang, L. C. Tsui, J. R. Riordan, and J. W. Hanrahan. 1993. Multi-ion pore behavior in the CFTR chloride channel. *Nature.* 366:79–82.

Takeshima, H., S. Nishimura, T. Matsumoto, H. Ishida, K. Kangawa, N. Minamino, H. Matsuo, M. Ueda, M. Hanaoka, T. Hirose, and S. Numa. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature.* 339:439–445.

Tao, T., J. Xie, M. L. Drumm, J. Zhao, P. B. Davis, and J. Ma. 1996a. Slow conversions among subconductance states of cystic fibrosis transmembrane conductance regulator chloride channel. *Biophys. J.* 70:743–753.

Tao, T., J. Xie, M. L. Drumm, J. Zhao, P. B. Davis, and J. Ma. 1996b. Modulation of CFTR channel by membrane surface charges. *Biophys. J.* 70:A72.

Tilly, B. C., M. C. Winter, L. S. Ostegaard, C. O'Riordan, A. E. Smith, and M. J. Welsh. 1992. Cyclic AMP-dependent protein kinase activation of cystic fibrosis transmembrane conductance chloride channels in planar lipid bilayers. *J. Biol. Chem.* 267:9470–9473.

Tsui, L. C. 1995. The cystic fibrosis transmembrane conductance regulator gene. *Am. J. Respir. Crit. Care Med.* 151:S47–S53.

Tu, Q., P. Velez, M. Cortes-Gutierrez, and M. Fill. 1994. Surface charge potentiates conduction through the cardiac ryanodine receptor channel. *J. Gen. Physiol.* 103:853–867.

Welsh, M. J., and A. E. Smith. 1993. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell.* 73:1251–1254.

Xie, J., M. L. Drumm, J. Ma, and P. B. Davis. 1995. Intracellular loop between transmembrane segments IV and V of cystic fibrosis transmembrane conductance regulator is involved in regulation of chloride channel conductance state. *J. Biol. Chem.* 270:28084–28091.