Slow Conversions among Subconductance States of Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel

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ABSTRACT The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel exhibits multiple subconductance states. To study the regulation of conductance states of the CFTR channel, we expressed the wild-type CFTR protein in HEK 293 cells, and isolated microsomal membrane vesicles for reconstitution studies in lipid bilayer membranes. A single CFTR channel had a dominant conductance of 7.8 pS (H), plus two sub-open states with conductances of ~6 pS (M) and 2.7 pS (L) in 200 mM KCl with 1 mM $MgCl_2$ (intracellular) and 50 mM KCl with no $MgCl_2$ (extracellular), with pH maintained at 7.4 by 10 mM HEPES-Tris on both sides of the channel. In 200 mM KCl, both H and L states could be measured in stable single-channel recordings, whereas M could not. Spontaneous transitions between H and L were slow; it took 4.5 min for L→H, and 3.2 min for H→L. These slow conversions among subconductance states of the CFTR channel were affected by extracellular Mg; in the presence of millimolar Mg, the channel remained stable in the H state. Similar phenomena were also observed with endogenous CFTR channels in T84 cells. In high-salt conditions (1.5 M KCl), all three conductance states of the expressed CFTR channel, 12.1 pS, 8.2 pS, and 3.6 pS, became stable and seemed to gate independently from each other. The existence of multiple stable conductance states associated with the CFTR channel suggests two possibilities: either a single CFTR molecule can exist in multiple configurations with different conductance values, or the CFTR channel may contain multimers of the 170-kDa CFTR protein, and different conductance states are due to different aggregation states of the CFTR protein.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is composed of two motifs, each containing a membrane-spanning domain (MSD) and a nucleotide-binding domain (NBD), which are linked by an intracellular regulatory (R) domain (Riordan et al., 1989). This 170-kDa protein constitutes a linear conductance Cl^- channel (Anderson et al., 1991a; Bear et al., 1992) that is regulated by cAMP-dependent protein kinase (PKA) phosphorylation at multiple sites located in the R domain (Tabcharani et al., 1991; Cheng et al., 1991; Rich et al., 1991; Chang et al., 1993), and binding and hydrolysis of ATP by the NBDs (Anderson et al., 1991b; Quinton and Reddy, 1992; Hwang et al., 1994). Mutations in the gene coding for CFTR are responsible for the lethal genetic disease cystic fibrosis (Quinton, 1990; Tsui, 1992; Welsh and Smith, 1993; Riordan, 1993; Gadsby and Nairn, 1994).

Under close to physiological conditions, a single CFTR channel has a linear Cl^- conductance of 7–10 pS (Egan et al., 1992; Tabcharani et al., 1993; Gunderson and Kopito, 1994). Like many other ion channels, the CFTR Cl^- channel exhibits distinct subconductance states, which were observed in both patch-clamp recordings (Haws et al., 1992; McDonough et al, 1994) and planar bilayer reconstitutions (Xie et al., 1995), but they have received relatively little attention until recently (Gunderson and Kopito, 1995). The mechanism underlying the multiple conductance states of the CFTR channel is largely unknown.

In this study, we focus on the distribution of subconductance states associated with the CFTR Cl^- channel reconstituted in lipid bilayer membranes. We found that both endogenous and expressed CFTR channels contained at least three stable open configurations, with distinct conductance values.

MATERIALS AND METHODS

Expression of CFTR in HEK 293 cells

The wild-type CFTR cDNA (Drumm et al., 1990) was subcloned into the eukaryotic expression vector pCEP4 (Invitrogen) between the *Nhe*I and *Xho*I restriction sites. A human embryonic kidney cell line (HEK 293-EBNA; Invitrogen) was used for the transfection and expression of CFTR protein. The cell line contains a vector pCMV-EBNA, which constitutively expresses the Epstein-Barr virus (EBV) EBNA-1 gene product and increases the transfection efficiency of EBV-based vectors. The parent cell line was 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 the cell culture media to maintain selection of the cells containing pCMV-EBNA vector until after the CFTR gene transfer. pCEP4(CFTR) was then introduced to the cell using the lipofectin reagent (Life Technologies) according to the manufacturer's instructions. Two days after transfection, the cells were passed and selected for hygromycin resistance (hygromycin B, 260 μg/ml). Three weeks after transfection, microsomal vesicles were isolated from transfected cells. The expression of CFTR protein was confirmed by Western blot using antibody against the R domain of CFTR (MAb 13-1, Genzyme) (Xie et al., 1995).

Received for publication 19 July 1995 and in final form 15 November 1995.
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0006-3495/96/02/743/11 $2.00

Isolation of microsomal membrane vesicles from T84 cells

T84 cells were grown in DMEM: Ham's F12 medium (1:1 ratio) with 5% fetal bovine serum and 2 mM glutamine, 50 μg/ml penicillin and 50 μg/ml streptomycin. The microsomal membrane vesicles from the T84 cells were isolated following the procedure of Tilly et al. (1992). Briefly, cells were homogenized in a buffer containing 250 mM sucrose, 2 mM EDTA, 10 mM HEPES-Tris (pH 7.4) plus protease inhibitors. The nuclei and mitochondria were removed by centrifugation at 4500 × *g*. The resulting membrane vesicles were pelleted by centrifugation at 26,000 × *g* and resuspended in 150 mM KCl, 10 mM HEPES-Tris (pH 7.2) at a protein concentration of 6–8 mg/ml. The membrane vesicles were stored at −75°C until use.

Isolation of microsomal membrane vesicles from HEK 293 cells

Microsomal vesicles were isolated from HEK 293 cells expressing the wild-type CFTR protein, using a modified protocol of Gunderson and Kopito (1994). Briefly, 12 75-cm^2 flasks of 293 HEK cells transfected with pCEP4(CFTR) vectors were harvested. The cell pellet was resuspended in ice-cold hypotonic lysis buffer (10 mM HEPES, pH 7.2, 1 mM EDTA, 5 μM diisopropyl fluorophosphate, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 mg/ml benzamidine) before lysis by 10 strokes in a tight-fitting Dounce homogenizer, followed by 15 strokes after the addition of an equal volume of sucrose buffer (500 mM sucrose, 10 mM HEPES pH 7.2). Microsomes were collected by centrifugation of postnuclear supernatant (600 × *g*, 15 min) at 100,000 × *g* for 45 min and resuspended with 1.0 ml prephosphorylation buffer (250 mM sucrose, 10 mM HEPES, pH 7.2, 5 mM Mg-ATP, and 100 units/ml PKA catalytic subunit). The membrane vesicles were stored at a protein concentration of 2–6 mg/ml at −75°C until use. Usually, 1–3 μl of microsomal membrane vesicles was added to the *cis* solution (1 ml volume) for the incorporation of CFTR channels.

Planar bilayer reconstitution of CFTR channels

Lipid bilayer membranes were formed across an aperture of ~200 μm diameter with a lipid mixture of phosphatidylethanolamine:phosphatidylserine:cholesterol in a ratio of 6:6:1; the lipids were dissolved in decane at a concentration of 40 mg/ml (Ma and Zhao, 1994). Unless otherwise specified, the recording solutions contained: *cis* (intracellular), 200 mM KCl, 2 mM ATP, 1 mM $MgCl_2$, and 10 mM HEPES-Tris (pH 7.4); *trans* (extracellular), 50 mM KCl, 10 mM HEPES-Tris (pH 7.4). Microsomal vesicles (1–3 μl) containing wild-type CFTR proteins were added to the *cis* solution. Fifty units/ml of PKA catalytic subunit (Promega) was always present in the *cis* solution. The experimental conditions selected only CFTR channels that were oriented in the bilayer membrane in the *cis*-intracellular, *trans*-extracellular manner.

To maintain stability of the bilayer membrane and the CFTR channel activities, designed pulse protocols were used to measure currents through the single CFTR channels. The bilayer membrane was kept at a holding potential of 0 mV and pulsed to different test potentials of either 2-s or 5-s duration. The interval between consecutive episodes was 10 s.

Single-channel currents were recorded with an Axopatch 200A patch-clamp unit (Axon Instrument). Data acquisition and pulse generation are 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 50 Hz, through an 8-pole Bessel filter. Single-channel data analyses were performed with pClamp software (Axon Instrument).

RESULTS

Endogenous CFTR channels in T84 cells

T84 is a human colonic epithelial cell line that expresses abundant quantities of endogenous CFTR proteins (Cliff and Frizzell, 1990). When microsomal membrane vesicles isolated from T84 cells were fused with the lipid bilayer membrane, a linear conductance Cl^- channel with properties of CFTR was measured (Fig. 1 *A*). This channel exhibited the following properties: 1) in a KCl concentration gradient of 200 mM (*cis*)/50 mM (*trans*), the channel had a dominant conductance of 7.8 ± 0.6 pS, with a reversal potential of +22.4 ± 1.4 mV (Fig. 1 *C*, □); 2) opening of the channel strictly required the presence of ATP (2 mM) and PKA (catalytic-subunit, 50 units/ml) in the *cis* solution; and 3) the channel was sensitive to blockade by diphenylcarboxylate (DPC) (data not shown). A further test was based on the interaction of exogenous R domain protein (RDP) with the CFTR channel (Ma et al., manuscript submitted for publication). The 32-kDa polypeptide of RDP, corresponding to the entire exon 13 plus 85 base pairs of exon 14 of human CFTR cDNA, was expressed in a TNT-coupled reticulocyte lysate system. When added to the intracellular solution, the unphosphorylated RDP consistently blocked this 8-pS Cl channel (Ma et al., manuscript submitted for publication).

The reconstituted CFTR channel was stable in the bilayer membrane. Usually it lasted for 45–60 min without appar-

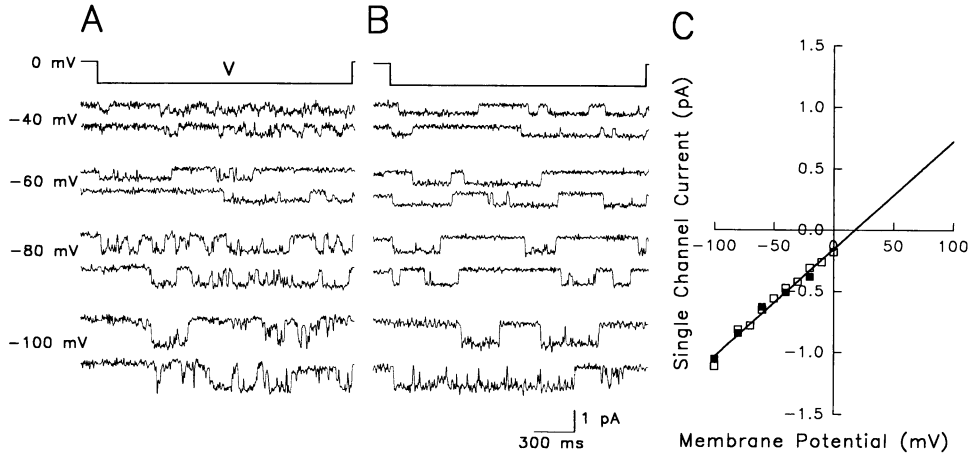

FIGURE 1 CFTR channels in T84 and HEK 293 cells. Selected single-channel currents at the given potentials were taken from T84 cells (*A*, bilayer 93318) and HEK 293 cells (*B*, bilayer 95504). The current-voltage relationship is represented in *C*. □, T84 cells; ■, HEK 293 cells. The solid line is the best fit to all data points, with a slope conductance of 7.8 ± 0.6 pS and a reversal potential of +22.4 ± 1.4 mV.

ent rundown of channel activity. Although the channels in these experiments had a dominant conductance of 8 pS, we also observed subconductance states, as shown in Fig. 2. With repetitive test pulses to −80 mV, the channel initially displayed a dominant conductance of ~8 pS (mean current amplitude of −0.72 pA at −80 mV). Eight minutes later, the channel suddenly converted from 8 pS to a lower conductance state (2–3 pS) (see the fifth episode in Fig. 2). Similar phenomena were observed in three other experiments, that is, a single CFTR channel would change its conductance state during the later stage of the experiment. This low conductance channel (2–3 pS) appeared to be CFTR, because we never saw the additive combination of 11 pS once a single 7.8-pS CFTR channel had been observed in the bilayer membrane.

However, systematic study of the low-conductance state associated with the endogenous CFTR channel was difficult because of an intrinsic low-conductance Cl^- channel present in T84 cells (Fig. 3). This low-conductance Cl^- channel had a number of properties that were distinct from those of the CFTR channel (see Table 1). First, the channel had a linear conductance of 3.2 pS (Fig. 3 *B*), which was similar to the low-conductance Cl^- channel identified in nonepithelial cells (Gabriel et al., 1992). Second, the 3.2-pS channel could be blocked by 500 μM DIDS (Fig. 3 *A*), but not by DPC (up to 3 mM). In addition, the exogenous R domain protein, which consistently blocked the CFTR channel, did not show any effect on the 3.2-pS Cl channel (Fig. 3 *A*).

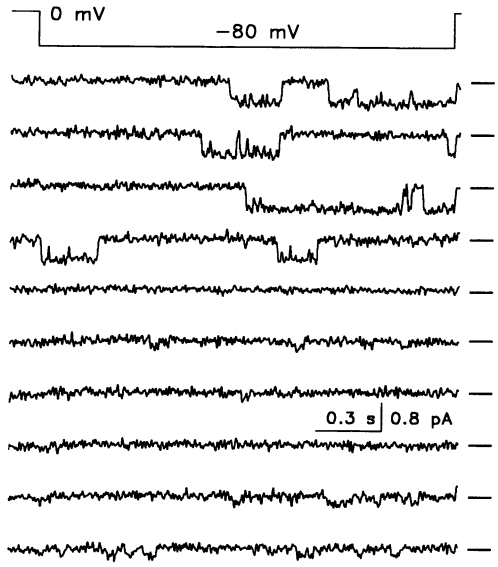

FIGURE 2 Transition from 8 pS to lower conductance of the endogenous CFTR channel. Consecutive episodes of a single CFTR channel obtained from T84 cells were measured with test pulses to −80 mV from a holding potential of 0 mV (bilayer 94829). The beginning episode was taken 7 min after the channel was incorporated into the bilayer membrane. Note the conversion from high to low conductance states, starting from the sixth episode. This spontaneous conversion was observed in three other experiments with the endogenous CFTR channel in T84 cells.

CFTR expressed in HEK 293 cells

To further characterize the function of the CFTR channel, we studied CFTR from a heterologous expression system. CFTR was expressed by transfecting the CFTR cDNA under CMV control into a human embryonic kidney cell line (Xie et al., 1995). The HEK 293 cells had high efficiency of transfection mediated by the lipofectin reagent (Felgner et al., 1987). In addition, the low-conductance Cl^- channel (2–3 pS), which complicated interpretation of the subconductance states of CFTR from T84 cells, was not observed in microsomal vesicles from untransfected HEK 293 cells (n = 21).

After expression of the CFTR protein, microsomal membrane vesicles were isolated and incorporated into the lipid bilayer membrane for the measurements of single CFTR channel activities. The heterologously expressed CFTR channels (Fig. 1 *B*) were similar to the endogenous CFTR channels in T84 cells. As shown in Fig. 1 *C*, the dominant conductance state of the expressed CFTR channel was nearly identical to the endogenous CFTR channel (open squares, T84 cells; filled squares, HEK 293 cells). Both channels had slow kinetics of gating, with mean open lifetimes of τ_{O1} ~25 ms (fast gating) and τ_{O2} ~150 ms (slow gating) at −80 mV (Fig. 4). The distribution of fast and slow open events, however, was significantly different for the endogenous and expressed CFTR channels. The relative occurrence of fast open events (yo1/yo2) was 1.38 in T84 cells and 0.12 in HEK 293 cells. Thus, the endogenous CFTR channels contained more fast open events.

The mechanism underlying the difference between the endogenous and expressed CFTR channels is not clear, but other regulatory molecules present in the vesicles could account for the different gating properties between the endogenous and the expressed CFTR channels. The difference in lipid environment, the putative interaction with other membrane proteins, or the self-interaction among CFTR molecules could all possibly alter the function of the CFTR channel. Certainly, the HEK 293 cells overexpressed CFTR protein. If CFTR molecules interact with each other, the functional properties of the Cl^- channel may likely be different in this system (personal communication with Dr. J. Wine).

Subconductance states of CFTR channels

Close examination of the channel recordings revealed several subconductance states of CFTR. In addition to the full conductance state (7.8 pS, H), at least two sub-open levels with conductances of ~6 pS (M) and 2.7 ± 0.4 pS (L) could be clearly identified, in both T84 and HEK 293 cells (Fig. 5). The three conductance levels (H, M, and L) interconvert with each other on a time scale of milliseconds.

A representative experiment with a CFTR channel expressed in HEK 293 cells was shown in Fig. 6. The channel had an initial conductance of 8 pS (H), which converted to the L state (starting from episode 24), then converted back

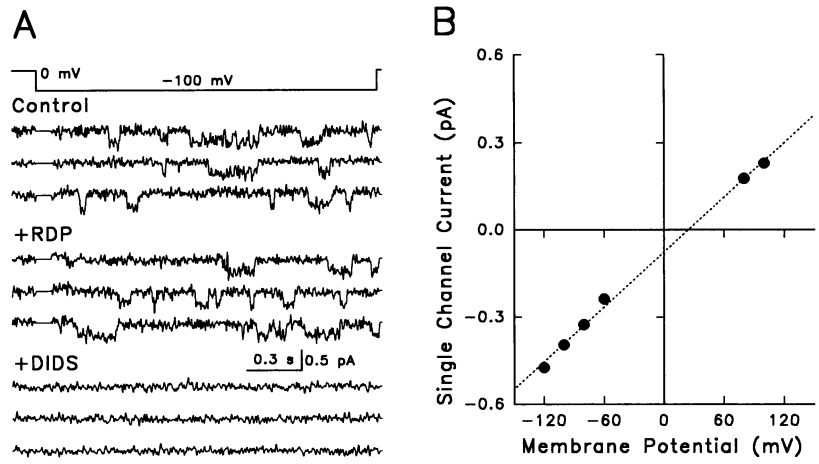

FIGURE 3 A small conductance Cl^- channel in T84 cells. The small conductance Cl^- channel was frequently observed in T84 cells ($n > 20$) (*A*) (bilayer 94831). The channel was insensitive to the exogenous R domain protein added to the *cis* solution (+RDP), and it could be completely blocked by 500 μM DIDS added to both solutions (+DIDS). The channel had a slope conductance of 3.2 ± 0.8 pS, with a reversal potential of +24.5 ± 2.8 (in 200 mM KCl (*cis*)/50 mM KCl (*trans*)) (*B*).

to the H state (starting from episode 48). The spontaneous conversion from H to L was observed in 58 of 82 experiments in which an 8-pS CFTR was first incorporated into the bilayer membrane. Of the 58 experiments, 42 channels showed recovery of the H state, i.e., conversion from L back to H; the other 16 channels remained in the L state for the duration of the experiment. Usually, all three conductance states (H, M, and L) were observed for a single CFTR channel.

With the expressed CFTR channel in HEK 293 cells under the basal condition of 200 mM KCl (*cis*)/50 mM KCl (*trans*), both H and L states could be measured in stable single-channel recordings ($n = 39$ for H; $n = 26$ for L). In contrast, we rarely observed a CFTR channel that existed in stable intermediate (M) state ($n = 2$). The following additional evidence suggests that the L state is closely related to the CFTR protein. First, Cl^- channels with a conductance of ~3 pS were never observed in untransfected HEK 293 cells ($n = 21$). We did encounter a high-conductance Cl^- channel (102 ± 14 pS, $n = 9$), which was probably an endogenous Cl^- channel located in the endoplasmic reticulum (ER) membranes of the HEK 293 cells, as it was observed in both microsomal vesicles and ER membrane fractions isolated in HEK 293 cells, independently of transfection with CFTR cDNA. Second, the 2.7 pS (L) Cl^- channel recorded in HEK 293 cells transfected with CFTR cDNA was sensitive to blockade by DPC, similar to the 8 pS CFTR channel (Figs. 9 and 10). Thus, the L state was different from the endogenous 3.2 pS Cl^- channel measured in T84 cells (Fig. 3; see Table 1). Third, in a KCl concentration gradient of 200 mM (intracellular)/50 mM (extracellular),

TABLE 1 Low-conductance Cl^- channels in T84 and HEK 293 cells

	G (pS)	V_{rev} (mV)	DIDS	DPC
T84 cells	3.2 ± 0.8	24.5 ± 2.8	+	−
CFTR-transfected HEK 293 cells*	2.7 ± 0.6	25.7 ± 1.8	−	+

*No such channel is observed in untransfected HEK 293 cells. This channel is also observed in T84 cells when the 8-pS CFTR channel is seen.

the L state had a reversal potential of $V_{rev} = +25.7 \pm 1.8$ mV (Fig. 7 B), similar to that of the H state ($V_{rev} = +22.4 \pm 1.4$ mV) (Fig. 1 *C*). Thus, the L state is also a chloride-selective channel.

The single-channel currents were measured with a cut-off frequency of 50 Hz, which limited the time resolution of the bilayer system to 1.4 ms, i.e., open or closed events with durations less than 1.4 ms would not be detected (Colquhoun and Sigworth, 1983). Thus, it is possible that the three conductance levels (H, M, and L) we observed underestimate the conductance states associated with the CFTR channel.

Slow conversions among the subconductance states

The experiment shown in Fig. 7 further supports the observation that the CFTR channel can exist in two stable conductance states (H and L) at 200 mM KCl, 1 mM $MgCl_2$ (*cis*)/50 mM KCl, 0 $MgCl_2$ (*trans*). Here, a Cl^- channel with low conductance state (L) converted spontaneously to the 8-pS Cl^- channel (H) 7 min after incorporation into the bilayer membrane. The transition from L to H involved the same CFTR channel, because the occurrence of H followed the disappearance of L (Fig. 7). Such spontaneous conversion from a low- to a high-conductance state of CFTR channel was observed in multiple experiments ($n = 56$). The existence of both H→L (Fig. 6) and L→H (Fig. 7) transitions, sometimes several times in the same experiment, argues against the possibility that rundown or degradation of the CFTR channels in the bilayer membrane accounts for the subconductance states we observed.

The time it took for a single CFTR channel to convert from L to H (Table 2), or from H to L (Table 3), varied from experiment to experiment. For L→H, the average transition time was 281 ± 323 s (mean ± SD), whereas for H→L, it was 191 ± 185 s. The fact that these estimates had standard deviations almost identical to the mean values suggests a possible Poisson distribution of the transition times. This

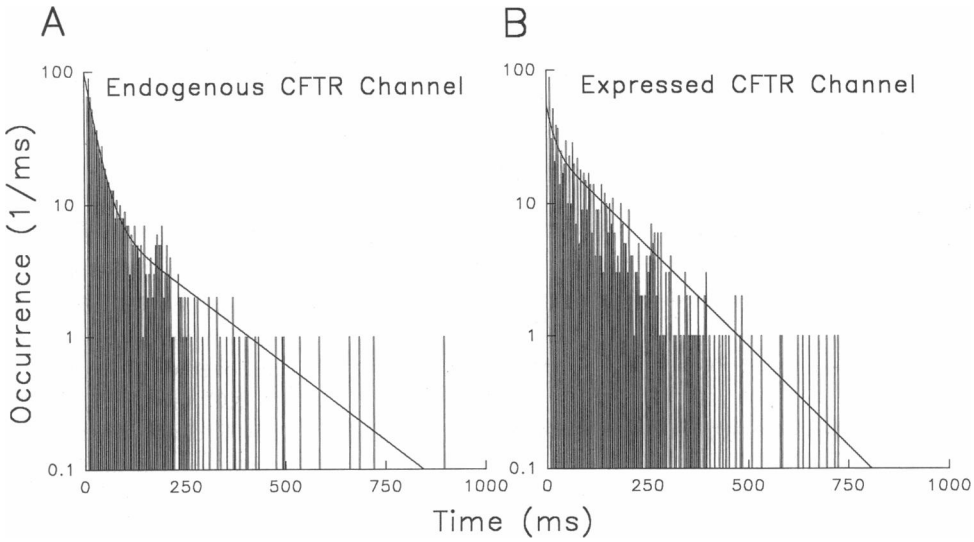

FIGURE 4 Open time histograms at −80 mV. Open events from single CFTR channels were calculated at −80 mV, in T84 cells (*A*) and HEK 293 cells (*B*). The histograms were constructed with a total of 3805 open events with the endogenous CFTR channel, and 4156 open events with the expressed CFTR channel. The solid lines represent the best fit according to $y = y1/\tau_{O1} \exp(-t/\tau_{O1}) + y2/\tau_{O2} \exp(-t/\tau_{O2})$, where τ_{O1} = 24.4 ms, τ_{O2} = 189.1 ms, y1/y2 = 2270/1648= 1.38 (*A*); and τ_{O1} = 15.6 ms, τ_{O2} = 144.6 ms, y1/y2 = 462/3850 = 0.12 (*B*).

was confirmed by the cumulative histogram analyses shown in Fig. 8, which plotted the occurrence of stable channels as a function of time after incorporation into the bilayer membrane. It is clear that the occurrence of channels in the H (Fig. 8 *A*) or L (Fig. 8 *B*) state decayed exponentially with time. The best-fit time constants were 222 s and 276 s, for H→L and L→H, respectively. This analysis suggests that the observed transitions among subconductance states of the CFTR channel represent a stochastic process that is likely an intrinsic property of the CFTR protein.

Effects of Mg^{2+} and perchlorate on CFTR channel

We tried several manipulations in an attempt to influence these spontaneous transition processes. Neither transition step (H→L or L→H) was sensitive to the free Mg^{2+} in the intracellular solution, over a concentration range of ~10 μM to 10 mM. The hydrolysis of intracellular ATP did not seem to be essential, as both transitions were observed when AMP-PNP was substituted for ATP. In the presence of 20 μM ATP and 2 mM AMP-PNP, the CFTR channel had increased open lifetimes, as previously reported (Hwang et al., 1994), but still underwent spontaneous transitions among the subconductance states. Thus, ATP binding or hydrolysis was unlikely to participate in the slow transition among subconductance states of the CFTR channel.

Perchlorate is a chaotropic anion that increases the movement of H_2O molecules in solution, thus altering subunit interactions within protein complexes (Luttgau et al., 1983). Early studies have shown that perchlorate could alter conductance states of the ryanodine receptor Ca^{2+} release channel in skeletal muscle (Ma et al., 1993; Percival et al., 1994). Perchlorate accelerated the conversion process of CFTR from H to L, and eventually locked the channel in the L state (Fig. 9). In the presence of 10 mM perchlorate, the CFTR channel always underwent irreversible transition from H to L (n = 5); this suggests that possible structural changes of channel protein may underlie changes in conductance state of the CFTR channel.

Several other laboratories also observed subconductance states associated with the CFTR channel, but they did not see them as frequently as we did in our experiments (personal communication with Drs. M. J. Welsh and J. Wine). Previous work with the CFTR single channels was all

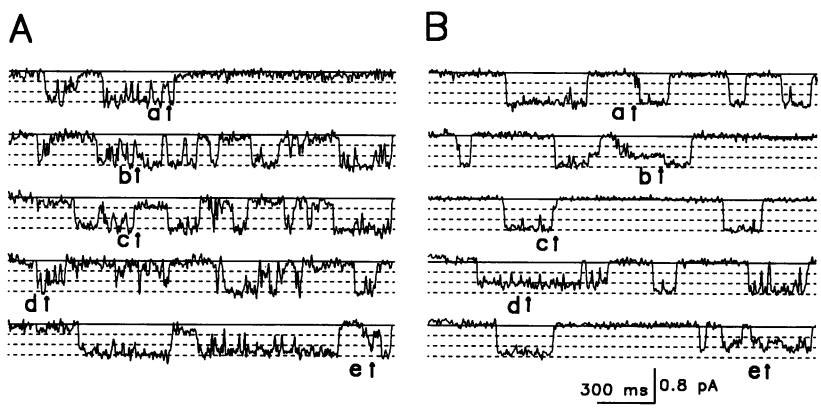

FIGURE 5 Subconductance states of the CFTR channel. Selected single channel currents at −80 mV for the endogenous CFTR channel (*A*) (bilayer 94124), and the expressed CFTR channel (*B*) (bilayer 94623). The dotted lines correspond to the three conductance levels of the Cl^- channel (H, M, and L). Fast transitions among the three conductance states can be clearly identified: (*a*) L→H; (*b*) M→H; (*c*) H→L; (*d*) H→M; (*e*) M→L.

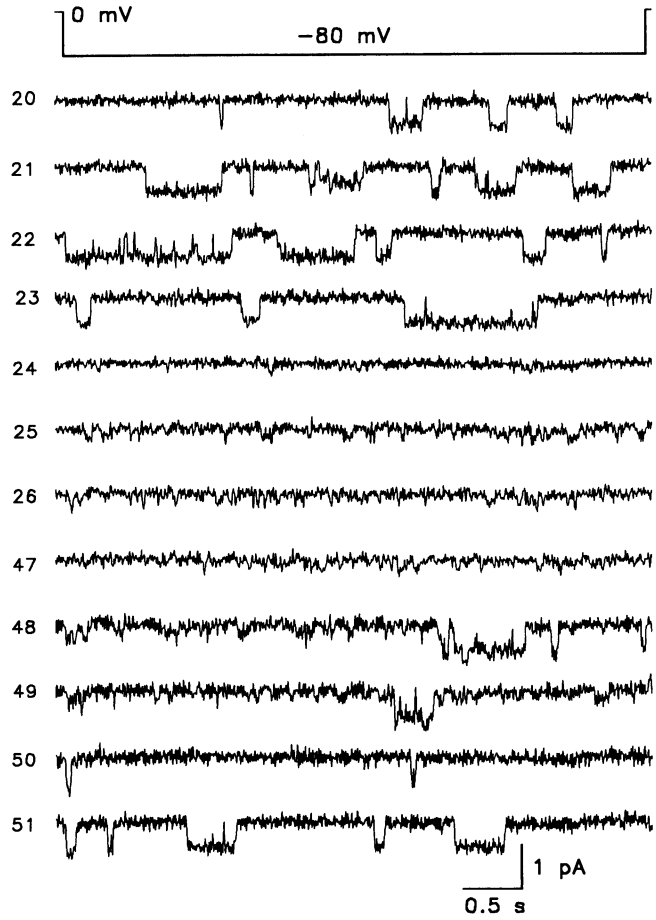

FIGURE 6 Transition from 8 pS to 2.7 pS of the expressed CFTR channel. The traces were taken from a representative experiment (bilayer 95525). The numbers on the left indicate the sequence of episodes taken after the incorporation of an 8 pS CFTR channel in the bilayer. Starting from episode 24, the channel converted from 8 pS to 3 pS. The channel stayed in the 3 pS state for 4 min, then it converted back to the 8 pS state. After that, transitions among the three conductance levels (H, M, and L) became frequent.

performed with Mg^{2+} present in both intracellular and extracellular solutions (patch-clamp measurements: Egan et al., 1992; Tabcharani et al., 1993; bilayer reconstitution studies: Bear et al., 1992; Gunderson and Kopito, 1994). Our studies thus far were all carried out with an extracellular solution that did not contain any divalent cations (*trans* solution: 50 mM KCl, 10 mM HEPES-Tris (pH 7.4)) (data shown in Figs. 1, 2, 7, and 8). When 2 mM $MgCl_2$ was added to the *trans* (extracellular) solution, we found that the channel could stay stable in the H state without conversion to lower conductance states (compare Fig. 10, *B* and *A*). The likelihood of a single CFTR channel to undergo spontaneous transition to lower conductance states was significantly less when Mg^{2+} was present in the extracellular solution. Without Mg, 58 of 82 CFTR channels underwent transition from H to L; with Mg^{2+} present, only 2 of 12 channels changed their conductance state from H to L. However, when the channel had converted to the L state, the addition of Mg^{2+} did not convert the channel to the H state ($n = 4$). This result suggests that Mg^{2+} in the extracellular solution plays a key role in regulating conductance states of the CFTR channel.

Conductance states of CFTR channel under high-salt conditions

In other studies when we tried to measure the conductance-activity relationship of the CFTR channel (Tao et al., manuscript submitted for publication), a new phenomenon was discovered. When the ionic composition was increased to 1 M KCl or higher, three distinct conductance levels of the CFTR channel were identified in stable single-channel recordings. The selected single-channel traces shown in Fig. 11 were all obtained at −80 mV test potential, under the identical ionic condition of 1.5 M KCl (*cis*)/0.5 M KCl (*trans*). The dotted lines correspond to the equally spaced current levels of −0.41 pA, −0.82 pA, and −1.23 pA at −80 mV. These different conductance levels probably correspond to the H, M, and L states of the CFTR channel measured at 200 mM KCl. The resulting current-voltage relationships had slope conductances of 12.1 ± 0.3 pS (▼, H), 8.2 ± 0.9 pS (●, M), and 3.6 ± 0.4 pS (■, L), respectively (Fig. 11, *right panel*).

The three conductance states were usually stable at the high-salt condition, i.e., once they occupied a given state, transitions to higher or lower states were rare (Fig. 11, *L state*). In 47 successful bilayer experiments at 1 M or 1.5 M KCl, we observed that 17 channels occupied the M state, 13 channels occupied the H state, 8 channels occupied the L state, and only 9 channels underwent measurable transitions among the three conductance states.

The results with the different conductance states of the CFTR channel are summarized in Table 4. Three main points need to be emphasized here. First, both fast (Fig. 5) and slow (Fig. 8) transitions among the subconductance states of the CFTR channel took place at the single-channel level. Second, the CFTR channel can exist in the three conductance states independently from each other, i.e., the L state did not necessarily have to convert to the H state, and vice versa. Third, the concentration of KCl seemed to influence the configuration of the CFTR proteins in the bilayer membrane, as the M state became stable at high-salt condition, whereas it was rarely seen at low-salt condition (compare 35% in 1.5 M KCl with <2% in 200 mM KCl).

Taken together, our data indicate that the CFTR channel can exist in at least three stable configurations, which seem to conduct Cl^- ions in a discrete manner.

DISCUSSION

We have shown that a single CFTR Cl^- channel can exist in multiple conductance states, at least three of which could be measured in stable single-channel recordings. The existence of more than one stable conductance state associated

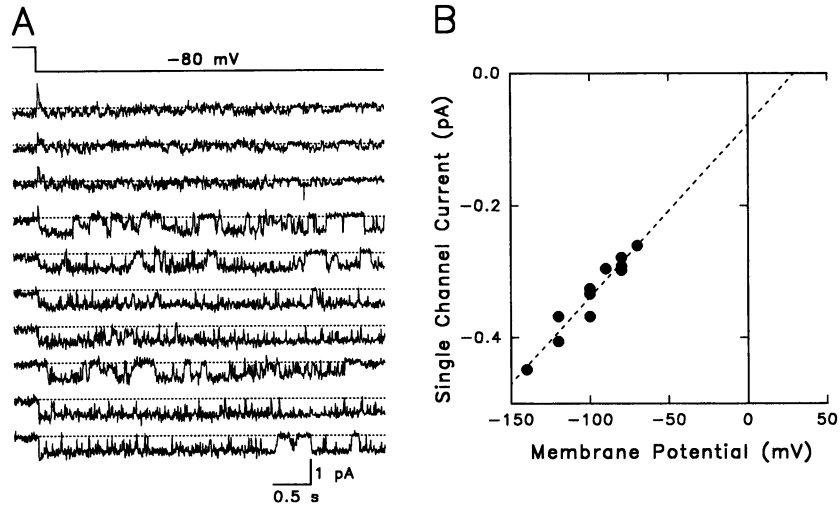

FIGURE 7 Transition from 2.7 pS to 8 pS of the expressed CFTR channel. (*A*) Consecutive current episodes were taken 6 min after the incorporation of a single CFTR channel into the bilayer membrane (bilayer 94O03). The channel started opening from a low-conductance state (2.7 pS), then converted to the high-conductance level (8 pS), starting from the fourth episode, approximately 8 min after the channel incorporation into the bilayer membrane. (*B*) The current-voltage relationship for the low-conductance state of the CFTR channel. The dotted line represents the best fit with a slope conductance of 2.72 ± 0.6 pS and a reversal potential of +25.7 ± 1.8 mV.

with the CFTR channel suggests that either a single CFTR molecule can exist in multiple configurations with distinct conductances for Cl^- ions, or the CFTR channel may contain multimers of the 170-kDa CFTR protein that aggregate or separate to form different conductance states of the Cl^- channel.

TABLE 2 Examples of conversion from L to H conductance states of CFTR channel

Experiment number	Transition time (s)	Final state
950403#1	990	L→L
950407#2	90	L→H+L
950411#4	1430	L→H→H+L
950413#2	40	L→H→H+L
950417#1	1020	L→L
950413#7	100	L→H
950417#2	40	L→H→H+L
950417#5	10	L→H→H+L
950417#7	100	L→H+L
950418#1	330	L→H→H+L
950418#2	90	L→H→H+L
950418#3	330	L→H→H+L
950424#1	40	L→H→H+L
950424#5	1280	L→L
950426#6	88	L→H
950426#7	144	L→H+L
950427#7	168	L→H+L
950428#1	72	L→H
950428#2	100	L→H
950428#5	280	L→H
Average (above experiments plus others)	281 ± 323	(*n* = 70)

The table only lists individual experiments performed during the month of April 1995. The third column lists the final destination of each channel: H, 8-pS conductance state, and H+L, coexistence of subconductance states. The data were obtained with 11 preparations of microsomal vesicles isolated from HEK 293 cells, after transfection with wild-type CFTR cDNA. The average transition time was based on all bilayer experiments (09/94–05/95) that started with a 3-pS (L) CFTR channel.

The existence of subconductance states is an important clue that quasi-stable configurations of the CFTR protein exist, either by intramolecular or intermolecular interactions. These different configurations may function differently not only with respect to the Cl^- transport properties of the channel, but with respect to CFTR's interaction with other proteins, and the transport of molecules other than Cl^- ion such as ATP (Al-Awqati, 1995). These non-chloride-transport functions may be as important as the chloride transport as we consider therapeutic strategies for cystic fibrosis. Knowledge of such inter- or intramolecular interactions may be important in designing truncated constructs of CFTR for gene therapy using vectors with limited packaging capacity, or for estimating the quantitative requirements for CFTR gene delivery for correction of all the functional defects in cystic fibrosis.

Possible multimeric structure of CFTR channel

Subconductance states are common to many other ion channels, in particular those with oligomeric structures. For example, the gap junction channel, which is a hexamer of connexins, contained up to six distinct conductance states (Spray, 1994). The sarcoplasmic reticulum Ca^{2+} release channel, which is a tetramer of ryanodine receptors, contained at least four conductance states, which seemed to space equally (Ma and Zhao, 1994). The CFTR Cl^- channel is distinct in that transitions among the different conductance states are slow. The conversions between H (8 pS) and L (2.7 pS) conductance states of CFTR channel had time constants of 3–5 min, instead of milliseconds for conversion among ryanodine receptor substates, or other ion channels. The facts that nearly 70% of the CFTR channels exhibited subconductance states and the presence of these subconductance states in both native (T84) and heterologously expressed (HEK 293) channels argue against the obligatory involvement of accessory proteins in regulating conductance states of the Cl^- channel. Rather, the slow conver-

TABLE 3 Examples of conversion from H to L conductance states of CFTR channel

Experiment number	Transition time (s)	Final state
950505#6	104	H→L→L+H
950508#4	550	H→L→L+H
950508#6	100	H→L+H
950509#3	128	H→L→L+H
950509#6	544	H→L+H
950509#7	20	H→L→L+H
950510#1	180	H→L
950510#4	112	H→L→L+H
950511#3	160	H→L
950512#5	40	H→L
950515#2	300	H→L+H
950515#3	40	H→L→L+H
950516#1	30	H→L→L+H
950516#3	180	H→L
950516#5	160	H→L→L+H
950517#6	160	H→L+H
950518#1	592	H→L→L+H
950518#4	88	H→L→L+H
950518#6	320	H→L+H
950519#1	544	H→L+H
950519#2	336	H→L→L+H
950519#3	150	H→L
950519#4	40	H→L+H
950522#1	80	H→L+H
950522#2	50	H→L→L+H
950522#3	152	H→L+H
950522#6	60	H→L+H
950522#8	160	H→L→L+H
Average (above experiments plus others)	191 ± 185	(n = 58)

Experiments performed during the month of May 1995 were listed. The average was based on all bilayer experiments (09/94–05/95) that started with the 8-pS CFTR channel (H) in 200 mM KCl (*cis*) and 50 mM KCl (*trans*). Twenty-four CFTR channels that remained stable in the H state during the entire bilayer experiment (>35 min) were not included in the average.

sions among the subconductance states are likely to be intrinsic properties of the CFTR protein.

Inferences about a possible oligomeric structure of the CFTR channel can be made from an analysis of the conductance states. This has been shown for the channel formed by the dodecapeptide alamethicin (Boheim, 1974; Hall et al., 1984). The alamethicin pore exhibits up to six conductance states, each formed by aggregation of dodecapeptide monomers. Transitions to the higher or lower conductance levels occur by uptake or release of one monomer at a time (Boheim, 1974). The fact that the three conductance states of the CFTR channel can, under some conditions, function independently of each other (Table 4), and the observation that the chaotropic anion perchlorate "locks" the channel in the low-conductance state, raise the possibility that the higher conductance states could be due to aggregation of the CFTR molecules. If the 2.7-pS conductance corresponds to a monomeric CFTR channel, one would predict using this model that a functional 8-pS Cl^- channel contains at least three subunits of the 170-kDa CFTR polypeptide. On the other hand, in high-salt condition, the low-conductance state is distinctly less than one-third of the high-conductance state (see Fig. 11), so a simple additive subunit model may not suffice.

The different domains of CFTR (MSD, NBD, and R) seem to interact with each other (Ostedgaard et al., 1994; Ciaccia et al., 1994), but biochemical studies from Marshall et al. (1994) indicate that the full-length CFTR protein does not form stable aggregates, i.e., the CFTR protein exists predominantly as monomers of the 170-kDa protein. Further studies are required to test whether a single 170-kDa CFTR molecule or multiple molecules of CFTR are required to form a functional Cl^- channel. In particular, do CFTR molecules, either wild-type or mutant forms, mix with each other in forming a functional Cl^- channel? If they do, could the H state of the CFTR channel represent a trimeric structure of the CFTR proteins? Understanding the stoichiometry of CFTR channel is important not only for structure-function purpose, but also for therapeutic purposes in cystic fibrosis.

Contribution of hydrophilic domains to CFTR function

It is likely that the hydrophilic domains of CFTR (the R domain, the NBDs, and the cytoplasmic loops) modulate permeation through the channel, via electrostatic or allosteric mechanisms. Several lines of evidence are consistent with this idea. First, deletion of portions of the R

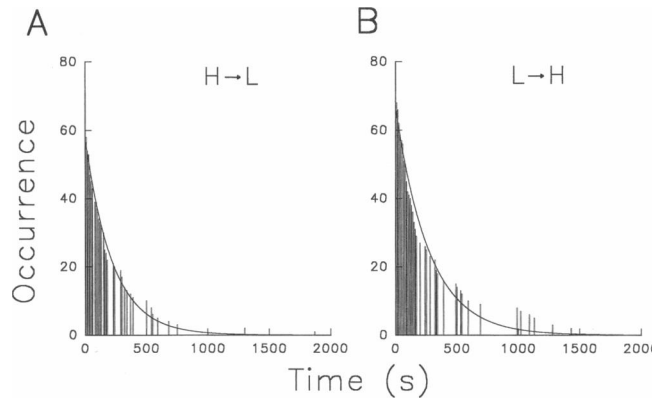

FIGURE 8 Histogram of transition times. To statistically analyze the spontaneous transitions between H and L states of the CFTR channel, the experiments were classified in two groups: (*A*) those channels started with a high conductance state, and (*B*) those channels started with a low conductance state. The histograms plotted the number of stable channels in H state (*A*) and L state (*B*) as a function of time after incorporation into the bilayer membrane. The total number of experiments was 58 in *A* and 70 in *B*. A total of 24 experiments where the CFTR channel stayed stable in the H state were not included in *A*. The solid lines are the best fits according to an exponential decay function, with time constants of τd = 222 ± 7 s for H→L (*A*) and 276 ± 12 s for L→H (*B*), respectively. Although the histogram in *B* could be better fitted with double exponentials, with the limited amount of data points, further quantitative analysis is not possible.

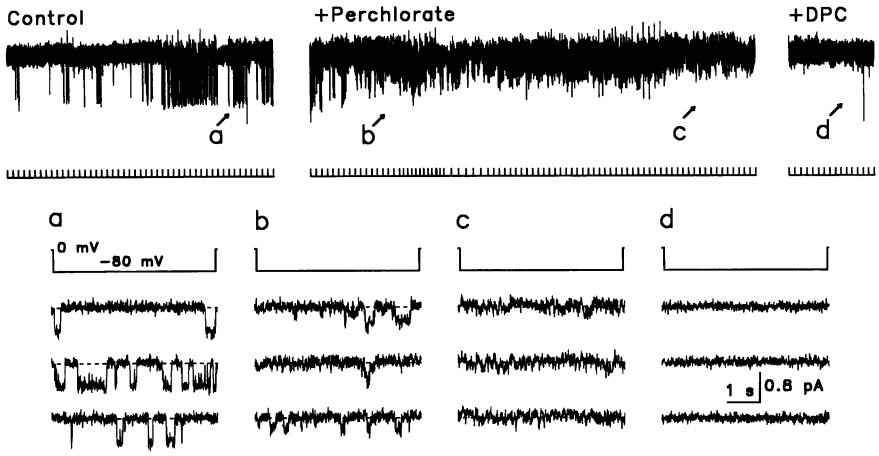

FIGURE 9 Effect of perchlorate on CFTR channel. Raw data from a complete experiment are represented in the upper traces. After incorporation of a CFTR channel, the bilayer membrane was held at a constant potential of 0 mV, and repetitively pulsed to −80 mV. Each episode consisted of a 5-s test pulse to −80 mV preceded by 0.06 s at 0 mV (pulse protocol shown in *a*, *b*, *c*, and *d*). The interpulse interval was 10 s; the period between test pulses at 0 mV is not shown in the figure. This channel stayed stable in the 8 pS state for ~8 min without apparent conversion to lower conductance states (control, *a*). Within 2 min after the addition of perchlorate (10 mM) to the *cis* solution (+perchlorate), the channel changed its conductance to ~6 pS (*b*), and eventually to ~3 pS. In the presence of perchlorate, the only stable conductance state of the CFTR channel was ~3 pS (*c*). All open events could be completely blocked by 3 mM DPC added to the *trans* solution (+DPC, *d*). Bilayer 95616/7. The traces were representative of four other experiments.

domain, or substantially altering its native charge, can result in a CFTR open channel that does not require PKA phosphorylation (Rich et al., 1991, 1993). Our groups have found that addition of exogenous unphosphorylated R domain protein to the cytoplasmic side of the channel results in channel closure, but once the exogenous R domain protein is phosphorylated, it is ineffective in closing the channel (Ma et al., manuscript submitted for publication). In addition, Xie et al. (1995) have shown that the intracellular loop joining transmembrane segments IV and V is involved in regulation of the conductance state of the Cl^- channel. A CFTR mutant in which 19 amino acids in this intracellular loop are deleted displays stabilization of the 6-pS (M) conductance state.

The intracellular loops of CFTR, most of them strongly hydrophilic, might display intramolecular interactions with other charged portions of the CFTR molecule, or intermolecular interactions with adjacent CFTR or other molecules. Such interactions would be expected to be disrupted or reduced in the presence of the chaotropic anion perchlorate, and under these conditions the CFTR channel exists almost entirely in the 3-pS low-conductance state, suggesting that

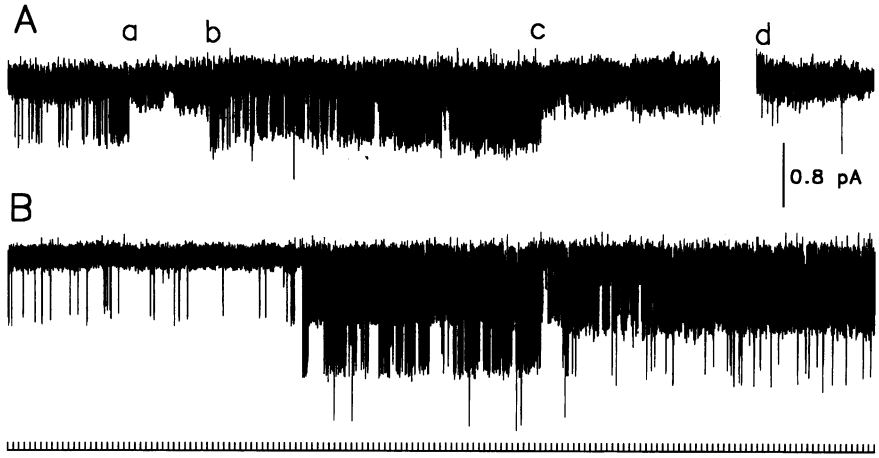

FIGURE 10 Effect of extracellular Mg^{2+} on the CFTR channel. Consecutive current episodes from two separate experiments are represented. The top traces were obtained with 1 mM $MgCl_2$ present in the *cis* solution and 0 mM $MgCl_2$ in the *trans* solution (*A*). The bottom trace was obtained with 2 mM $MgCl_2$ present in both *cis* and *trans* solutions (*B*). In the absence of extracellular Mg, the CFTR channel underwent spontaneous transitions from H to L (*a*), L to H (*b*), and then H to L (*c*). The channel was sensitive to blockade by 3 mM DPC added to the *trans* solution (*d*). With Mg^{2+} present in the extracellular solution, the channel stayed stable in the H state for the entire period of the experiment (>30 min, *B*). The traces shown in *b* are representative of 9 (of 11) other experiments. The marks at the bottom show the test pulse protocols used: each interval corresponds to one episode of recording at −80 mV (5-s duration); the time between two consecutive episodes was 10 s (same protocol as in Fig. 9).

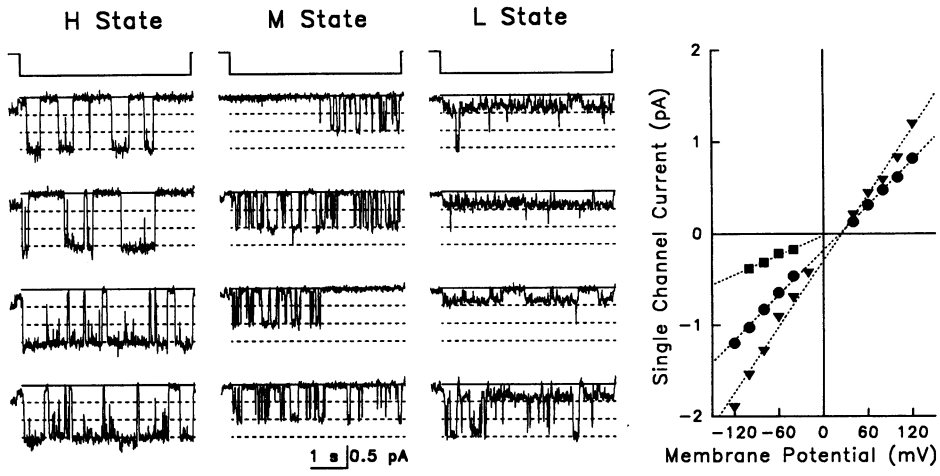

FIGURE 11 Multiple stable conductance states of CFTR channel in 1.5 M KCl. The selected single channel traces were obtained from three separate experiments (bilayers 95831/4, L state; 95831/6, M state; 95831/8, H state), at test potentials of −80 mV. The recording solutions were identical: *cis*, 1.5 M KCl, 1 mM $MgCl_2$, 2 mM ATP, 10 mM HEPES-Tris (pH 7.4), 50 units/ml C-subunit of PKA; *trans*, 0.5 M KCl, 10 mM HEPES-Tris (pH 7.4). The three distinct conductance states had slope conductances of 12.1 ± 0.3 pS (▼), 8.2 ± 0.9 pS (●), and 3.6 ± 0.4 pS (■), respectively. The corresponding reversal potentials were 24.7 ± 2.5 mV (▼), 22.3 ± 1.0 mV (●), and 5.9 ± 7.8 mV (■). The estimate of reversal potential for the L state had large standard error because we did not have enough data points with the L state of the CFTR channel.

interdomain or intermolecular interactions are most important in stabilizing the higher conductance states. The second intracellular loop may be important in stabilizing the H state (8 pS) over the M state (6 pS), for when a strongly hydrophilic portion of this loop is removed, the M state is favored, even at low salt concentrations (200 mM KCl) (Xie et al., 1995). It will be interesting to learn how the Δ19-CFTR channel behaves under high-salt conditions.

Role of extracellular Mg^{2+} and ATP hydrolysis to CFTR function

Our results show that Mg^{2+} in the extracellular solution plays important roles in stabilizing the conductance states of the CFTR channel (Fig. 10). The H state of the CFTR channel became less stable when Mg^{2+} is removed from the extracellular solution. In addition, a single CFTR channel exhibits clear inward rectification, when Mg^{2+} was absent from the extracellular solution (Zerhusen et al., manuscript submitted for publication). The linear I-V relationship of the CFTR channel could be restored through the addition of divalent cations (Ca^{2+} or Mg^{2+}) to the extracellular solution. It is possible that extracellular Mg^{2+} could regulate the interdomain interactions within the CFTR protein or alter the aggregation states of the CFTR proteins.

TABLE 4 Properties of the three stable conductance states of CFTR channel

	Ionic condition	H state	M state	L state
Conductance	0.2 M KCl	7.8 ± 0.6 pS	~6 pS	2.7 ± 0.3 pS
	1.5 M KCl	12.1 ± 0.3 pS	8.2 ± 0.9 pS	3.6 ± 0.4 pS
Experiments (number)	0.2 M KCl	39	2	26
	1.5 M KCl	13	17	8
Occurrence	0.2 M KCl	26%	1.3%	17%
	1.5 M KCl	28%	36%	17%

Only stable channels in a given conductance state were listed. The definition of stable channels was the continuous occupancy of the given state without conversion to other states for a period of 8 min or longer, as measured with three consecutive data files. Each data file contained 16 episodes of 5-s test pulses to different voltages, acquired at 10-s intervals. The total number of experiments performed was 152 with 0.2 M KCl, and 47 with 1.5 M KCl.

A recent study by Gunderson and Kopito (1995) showed that hydrolysis of ATP caused a fast conformational change of the CFTR channel accompanied by approximately a 15% change in the open conductance level (see also Fig. 11). The slow conversion process among the subconductance states of the CFTR channel observed in the present study is insensitive to the free Mg^{2+} in the intracellular solution, over a concentration range of ~10 μM to 10 mM. The hydrolysis of intracellular ATP did not seem to be essential to the slow transition process, as the phenomenon persisted when AMP-PNP was substituted for ATP. Thus, the slow transition step among the conductance states of the CFTR channel is different from the fast conformational change associated with ATP hydrolysis reported by Gunderson and Kopito (1995).

The CFTR protein contains clusters of negatively charged amino acids on several extracellular loops joining the transmembrane segments (Riordan et al., 1989), which could constitute the putative binding sites for Mg^{2+}. Our data give rise to the hypothesis that extracellular loops of CFTR participate in both rectification and stabilization of conductance state of the Cl^- channel, which can be tested by studies using site directed mutagenesis.

We thank Dr. Marlene Hosey (Northwestern University) for the initial supply of PKA.

This work was supported by grants from National Institutes of Health (RO1HL/DK49003 and P30DK27651 (PBD), RO1DK45965 (MLD), and a Feasibility grant from P30DK27651 (JM). JM is a Established Investigator of the American Heart Association.

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