Conformation, Independent of Charge, in the R Domain Affects Cystic Fibrosis Transmembrane Conductance Regulator Channel Openings

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ABSTRACT The R domain of cystic fibrosis transmembrane conductance regulator (CFTR), when phosphorylated, undergoes conformational change, and the chloride channel opens. We investigated the contribution of R domain conformation, apart from the changes induced by phosphorylation, to channel opening, by testing the effect of the peptidyl-prolyl isomerase, cyclophilin A, on the CFTR channel. When it was applied after the channel had been opened by PKA phosphorylation, cyclophilin A increased the open probability of wild-type CFTR (from $P_o = 0.197 \pm 0.010$ to $P_o = 0.436 \pm 0.029$) by increasing the number of channel openings, not open time. Three highly conserved proline residues in the R domain, at positions 740, 750, and 759, were considered as candidate targets for cyclophilin A. Mutations of these prolines to alanines (P3A mutant) resulted in a channel unresponsive to cyclophilin A but with pore properties similar to the wild type, under strict control of PKA and ATP, but with significantly increased open probability ($P_o = 0.577 \pm 0.090$) compared to wild-type CFTR, again due to an increase in the number of channel openings and not open time. Mutation of each of the proline residues separately and in pairs demonstrated that all three proline mutations are required for maximal P_o. When P3A was expressed in 293 HEK cells and tested by SPQ assay, chloride efflux was significantly increased compared to cells transfected with wild-type CFTR. Thus, treatments favoring the *trans*-peptidyl conformation about conserved proline residues in the R domain of CFTR affect openings of CFTR, above and beyond the effect of PKA phosphorylation.

INTRODUCTION

The CFTR is composed of two motifs, each containing a membrane-spanning domain and an NBD, which are linked by an intracellular R domain (Riordan et al., 1989). The R domain of CFTR contains the consensus phosphorylation sites for cAMP-dependent PKA that are the basis for physiologic regulation of the CFTR channel (Cheng et al., 1991; Picciotto et al., 1992). This domain exerts both inhibitory and stimulatory influences on the channel function. When the R domain is unphosphorylated, channel openings are inhibited, as demonstrated by three pieces of evidence. In the wild-type CFTR channel, PKA-dependent phosphorylation is a prerequisite for channel openings (Anderson et al., 1991; Rich et al., 1993a; Cheng et al., 1991). Deletion of a portion of the R domain results in relief of inhibition, for the ΔR(708–835) CFTR channel can open without phosphorylation (Rich et al., 1991, 1993b). The unphosphorylated R domain, expressed as a protein and added to the intracellular side of the channel, results in channel closure (Ma et al., 1995). However, when the exogenous R domain is phosphorylated, channel openings are stimulated (Ma et al., 1997; Winter and Welsh, 1997). In addition to the onset of channel openings in wild-type CFTR when the R domain is phosphorylated, other evidence also indicates a direct stimulatory role for the R domain in channel openings. The ΔR(708–835) CFTR channel, though it opens without phosphorylation, has low open probability, which fails to increase with phosphorylation even though several phosphorylation sites are retained, suggesting that some stimulatory property of the R domain has been lost. Exogenous phosphorylated R domain increases the open probability of the ΔR(708–835) CFTR channel (Ma et al., 1997; Winter and Welsh, 1997), so the phosphorylation-related stimulation resides within the R domain. Dulhanty and Riordan (1994) have shown that the R domain of CFTR, expressed as a separate protein, undergoes a conformational change as assessed by circular dichroism, when it is phosphorylated. Cotten and Welsh (1997) showed that covalent modification of the R domain by the sulfhydryl-modifying reagent *N*-ethylmaleimide rapidly and irreversibly stimulated CFTR channel activity, but did not affect the ability of unphosphorylated R domain to inhibit channel function. These data, taken together, suggest that charge on the R domain or the conformational changes it produces is an important determinant of whether it functions in a stimulatory or inhibitory mode.

Considerable effort has been devoted to demonstrating that phosphorylation, or addition of negative charges, to the R domain, induces conformational changes in the R domain and promotes channel openings (Rich et al., 1993b; Xie et al., 1997). Another mechanism of altering protein conformation without altering charge is isomerization about pro-

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Abbreviations used: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; DPC, diphenylcarboxylate; HEK, human embryonic kidney; NBD, nucleotide binding domain; PKA, protein kinase A; P_o, open probability; R, regulatory; SPQ, 6-methoxyl-*N*-(3-sulfopropyl)quinolinium; WT, wild type.

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line residues; thus, this strategy allows separation of conformational effects from charge effects on CFTR channel function. In this study we investigated whether the conformation about three highly conserved proline residues in the R domain, P740, P750, and P759, affects channel openings. We took two approaches to this question. First, we treated CFTR captured in the planar lipid bilayer with cyclophilin A, a protein that promotes peptidyl bond isomerization about proline residues. Second, we mutated these three proline residues singly and together to study the resulting CFTR mutants in the planar lipid bilayer. Our data indicate that changes in R domain conformation about critical proline residues is important for the stimulation of channel openings, but not for relief of inhibition.

MATERIALS AND METHODS

Mutagenesis

A portion of CFTR cDNA containing the R domain was subcloned into a pAlter-1 (Promega, Madison, WI) vector. Site-specific mutations were constructed following the manufacturer's instructions using the following three mutagenesis oligonucleotides (showed 5′ to 3′, with mutated base underlined): P740A, G CTC AGA ATC TG*C* TAC TAA GGA CAG C (*Rsa*I enzyme restriction site is destroyed); P750A, G GCT GAT GCG AG*C* CAG TAT CGC CTC (*Bsr*I site is created); P759A/T757S, C CTG AAG CGT GG*C* CGG AG*A* GCT GAT (*Bsa*HI site is created and *Bsr*I site is destroyed). The mutant clone was identified with restriction enzyme digestion. Although mutation was intended to affect only proline residues, upon sequencing, an additional conservative mutation, T757S, was noted. Subsequently, the individual prolines were mutated in sequence, with and without the T757S mutation, and their function was examined. No difference in channel activity was produced for any mutant by the T757S substitution. Data presented in this paper for mutants containing P759A mutation are for constructs that also contain the unexpected T757S mutation. The single proline mutations P740 and P759 were made directly using a pCEP4(WT-CFTR) vector following the instructions of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutated fragments created using the pAlter-1 system were subcloned into the eukaryotic expression vector pCEP4 by substituting a fragment in pCEP4(WT-CFTR) between *Bst*1107I and *Pml*I restriction sites with the corresponding mutated fragment. The mutated clone was identified with restriction enzyme digestion and confirmed with DNA sequence analysis.

Cell culture

The pCEP4 plasmid containing wild-type and mutant forms of CFTR were transfected into 293 HEK (293-EBNA, InVitrogen, San Diego, CA) cells using the lipofectAMINE reagent (Xie et al., 1995; 1996). The parent cells were passaged 1:5 two days before transfection. One or two days after transfection, cells were used for immunoprecipitation/Western blot assay, SPQ experiments, isolation of membrane vesicles, and/or reconstitution studies of CFTR in the planar lipid bilayer.

Identification of CFTR

For immunoprecipitation/Western blot assay, transfected cells were lysed and supernatant (48,000 $\times$ *g*, 1 h, 4°C) containing 5 mg total protein treated with 2 μg monoclonal antibody directed against the C-terminal region of CFTR (mAb 24-1, Genzyme, Cambridge, MA). Protein complexes were then precipitated with 20 μl protein G-agarose beads, and the precipitate solubilized with gel sample buffer and subjected to SDS-PAGE electrophoresis and Western-blotted exactly as previously described (Xie et al., 1996). Blots were probed with mAb 24-1 and developed with goat anti-mouse IgG using chemiluminescent detection according to the manufacturer's recommendation (SuperSignal CL-HRP substrate system; Pierce, Rockford, IL).

SPQ assay of chloride transport

Chloride flux across the plasma membrane was measured by SPQ assay with 293 HEK cells transfected with pCEP4(P3A) exactly as described previously for wild-type CFTR and other mutants (Xie et al., 1995). Cells grown on coverslips were cultured for 2–3 days. The CFTR cDNA in pCEP4 was transfected into cells and cultured for 1–2 days. Untransfected 293 HEK cells display little chloride flux at the plasma membrane either with or without reagents that stimulate cAMP production. However, 293 HEK cells transfected with pCEP4(WT) display a 6- to 10-fold increase in chloride flux at the membrane with cAMP stimulation. A CFTR mutant with normal single channel activity, but which is not fully processed and does not reach the plasma membrane (e.g., a CFTR mutants with deletion in the second intracellular loop, Δ19 CFTR), gives no evidence of chloride transport in the SPQ assay (Xie et al., 1995). Thus, this assay assesses whether a given CFTR mutant is both active and resident in the plasma membrane. SPQ fluorescent dye was introduced into the cells by hypotonic loading. The experimental procedures for measuring the forskolin-stimulated chloride transport across the plasma membrane of 293 HEK cells have been described in previous studies (Xie et al., 1995).

Vesicle preparation

Six 75 cm^2 flasks of 293 HEK cells transfected with either pCEP4(WT) or CFTR mutants (P3A, P2A, P740A, P750A, P759A) vectors were harvested and lysed following the procedure described previously (Xie et al., 1995, 1996). Briefly, cells were scraped into ice-cold phosphate buffered saline (PBS) and lysed by hypotonic lysis and Dounce homogenization in the presence of protease inhibitors. After sedimentation of nuclei and mitochondria at 6000 $\times$ *g* for 20 min, the supernatant was sedimented at 100,000 $\times$ *g* for 45 min at 4°C. The microsomes were resuspended in 600 μl buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.2, and stored at $-$80°C before use.

Reconstitution of the single CFTR channel

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 (Xie et al., 1995; 1996). The recording solutions contained *cis* (intracellular): 200 mM KCl, 2 mM ATP-Mg, and 10 mM Hepes-Tris (pH 7.4); *trans* (extracellular): 50 mM KCl, 10 mM Hepes-Tris (pH 7.4). Membrane vesicles (3–6 μl) containing wild-type or mutant CFTR protein were added to the *cis* solution. In experiments with WT, P3A, P2A, P740A, P750, and P759A, *cis* solution also contains 50 units/ml cAMP-dependent protein kinase A catalytic subunit. In experiments with ΔR(708–835) or ΔNEG2(817–838) channels, no PKA was present in the *cis* solution.

To study the effect of cyclophilin A on the CFTR channel, the purified recombinant cyclophilin A proteins (purchased from Sigma, St. Louis, MO) were added to the *cis* solution (at final concentrations of 0.4–0.8 μM). The specificity of the effect of cyclophilin A was tested through prior application of cyclosporin A (6–12 μM, a specific antagonist for cyclophilin A) to the CFTR channel. For a complete experiment, the activity of a single CFTR channel (WT, P3A, ΔR, or ΔNEG2) was recorded for 5–8

min under control conditions to establish the stability of the channel activity in the bilayer membrane. Following the application of cyclosporin A or cyclophilin A to the *cis* solution, the channel activity was recorded for another 5–10 min. The average P_o of the CFTR channel was calculated 3 min after the addition of either cyclosporin A or cyclophilin A.

To facilitate kinetic analysis, channel recordings containing one channel were selected for analysis. Bilayer channel currents were recorded with an Axopatch 200A patch clamp unit (Axon Instruments, Foster City, CA). Data acquisition and pulse generation were performed with a 486 computer and 1200 Digidata A/D-D/A converter (Axon Instruments). The currents were sampled at 1–2.5 ms/point and filtered at 100 Hz. The analyses of single channel data were performed with pClamp software (Axon Instruments) and custom programs. Data presented in this paper were obtained from at least four different preparations of membrane vesicles isolated from 293 HEK cells transfected with CFTR proline mutants.

RESULTS

Cyclophilin A enhances activity of the wild-type CFTR channel

Cyclophilin A, a cytosolic receptor for immunosuppressant drug cyclosporin A (CsA), has the enzymatic activity of a *cis-trans* peptidyl-prolyl isomerase. Thus, it could affect the configuration of the prolines in a protein molecule. When applied to the cytosolic solution at a concentration of 0.6 μM, cyclophilin A enhanced the activity of the WT CFTR channel (Fig. 1 *A*); 3–6 min after the addition of cyclophilin A, the average P_o of the WT CFTR channel increased from

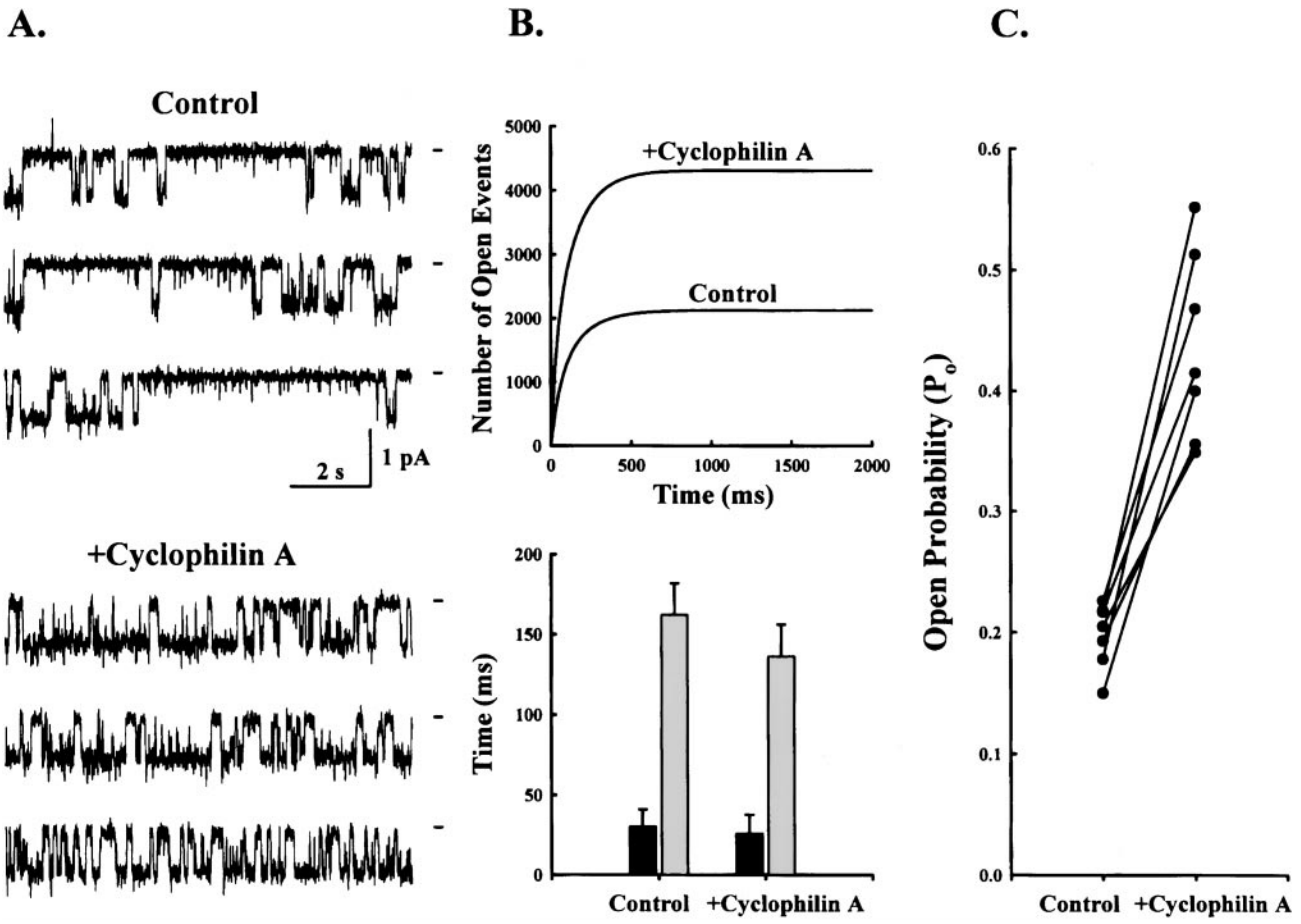

FIGURE 1 Effect of cyclophilin A on the WT CFTR channel. (*A*) Representative single channel current traces from a WT CFTR channel at −80 mV. The currents were measured with 2 mM ATP and 50 units/ml of PKA present in the *cis*-intracellular solution (*Control*); and 3 min after addition of cyclophilin A to the *cis* solution (+*Cyclophilin A*). Channel closed level is marked by the short bar on the right side of each current trace. (*B*) *Top panel:* cumulative open-time histograms at −80 mV for WT CFTR before and after the addition of cyclophilin A. Open events were analyzed with single channel currents recorded at 40 Hz cutoff filtering frequency; 16 data files with 20 min of total channel recording at −80 mV were used ($n = 7$ paired bilayer experiments) for WT CFTR channels before and after the application of cyclophilin A. A total of 2130 and 4315 open events were detected, respectively, for control and after the stimulation by cyclophilin A. The histograms were fitted with the following equation: $y = W_{01}(1 - \exp(-t/\tau_{01})) + W_{02}(1 - \exp(-t/\tau_{02}))$. The best-fit parameters were $W_{01} = 842.4$, $\tau_{01} = 56.1$ ms, $W_{02} = 1287.1$, $\tau_{02} = 162.1$ ms for control; $W_{01} = 1242.5$, $\tau_{01} = 29.4$ ms, $W_{02} = 3072.9$, $\tau_{02} = 142.8$ ms after stimulation by cyclophilin A. *Bottom panel:* kinetic analysis of WT CFTR channel before and after the stimulation by cyclophilin A. Channel open-time histograms from seven paired experiments were fitted separately by two exponentials. The averaged time constants τ_{01} (*black bar*) and τ_{02} (*gray bar*) were plotted before (*Control*) and after the addition of cyclophilin A (+*Cyclophilin A*). (*C*) Single channel open probability (P_o) was calculated at −80 mV from seven paired experiments with WT CFTR. The averaged values were $P_o = 0.197 \pm 0.010$ (*Control*), and $P_o = 0.436 \pm 0.029$ (+*Cyclophilin A*).

0.197 ± 0.010 to 0.436 ± 0.029 (n = 7) at −80 mV test potential (Fig. 1 *C*). This enhanced activity of the WT CFTR channel was due to specific enzymatic action of cyclophilin A with the CFTR protein, for pretreatment with cyclosporin A (10 μM), a specific antagonist for cyclophilin A, prevented the stimulatory effect of cyclophilin A on the WT CFTR channel (Fig. 2 *A*). Cyclosporin A, which also inhibits phosphatase 2B after binding to cyclophilin A (Fisher et al., 1998), had no effect on the WT CFTR channel (Fig. 2). This indicates that the bilayer system is free of phosphatase 2B. Cyclosporin A also reverses the stimulatory effect of cyclophilin A when applied after cyclophilin A, and cyclophilin A added before the application of PKA could not open the channel (data not shown).

The cyclophilin A-induced increase in WT CFTR channel activity resulted from an increase in the number of channel open events, not from changes in the open lifetime of the channel, as shown in the cumulative open-time histogram analyses (Fig. 1 *B*). Under control conditions (with 2 mM ATP and 50 units/ml PKA present in the cytosolic solution), the WT CFTR channel exhibited two open states with mean open lifetimes of τ_{o1} = 30.04 ± 10.90 ms and τ_{o2} = 161.9 ± 19.90 ms. After stimulation with cyclophilin A, the open lifetime constants remained essentially unchanged, with τ_{o1} = 25.82 ± 11.77 ms, τ_{o2} = 136.11 ± 19.99 ms. However, the average number of channel open events was increased by 2.48 ± 0.44-fold in the seven paired experiments.

Cyclophilin A fails to enhance the activity of the ΔR(708–835) CFTR channel

Cyclophilin A failed to stimulate the ΔR(708–835) CFTR channel (Fig. 3), which opens independently of PKA phosphorylation (Rich et al., 1991; Ma et al., 1997; Winter and Welsh, 1997). Compared with the WT CFTR channel, open probability of the ΔR(708–835) CFTR channel is significantly lower and unchanged with the application of cyclophilin A (Fig. 3 *B*). We speculated that cyclophilin A may act on proline residues in the deleted portion of the R domain, accounting for these results.

Of the six prolines in the deleted region (708–835) of ΔR CFTR, three (P740, P750, and P759) are highly conserved across species. We selected these prolines for further study. It was our hypothesis that if cyclophilin A stimulated opening of CFTR by virtue of promoting *cis-trans* isomerization about one or more of these proline residues, replacing these residues with alanines, which should favor an all-*trans* configuration, should eliminate the stimulatory effect of cyclophilin A. In addition, we speculated that all-*trans* mutant would have properties similar to cyclophilin A treated WT CFTR.

Reconstitution of the P3A CFTR chloride channel

By using site-directed mutagenesis we mutated all three prolines (P740, P750, and P759) into alanines, with the

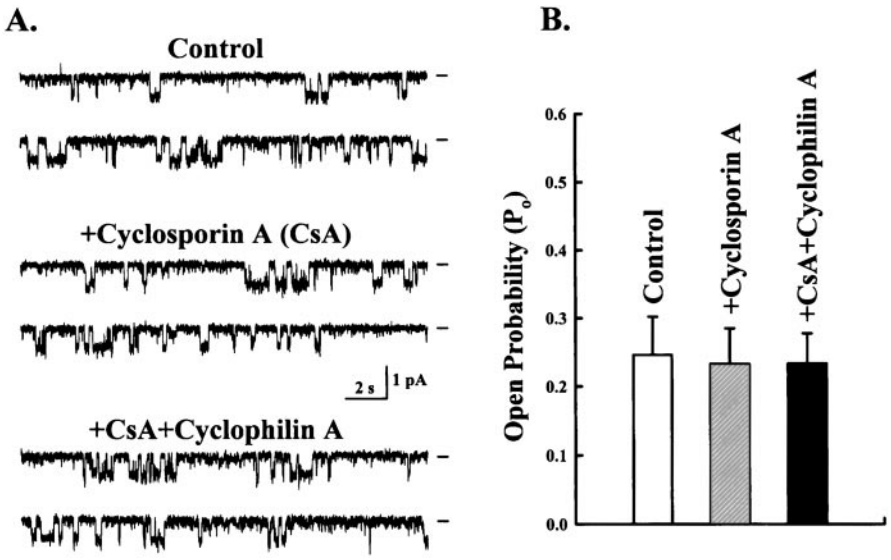

FIGURE 2 Cyclosporin A (CsA) prevents the effect of cyclophilin A on the WT CFTR channel. (*A*) The representative single channel current traces were recorded from a WT CFTR channel at −80 mV, with 2 mM ATP and 50 units/ml PKA present in the cytosolic solution (*Control*); 3 min after the addition of 10 μM cyclosporin A (+*Cyclosporin A*); followed by the addition of 0.6 μM cyclophilin A to the cytosolic solution (+*CsA*+*Cyclophilin A*). Note that activity of the channel did not change with the addition of cyclosporin A and subsequent addition of cyclophilin A. The short bar on the right side of each current trace indicates the channel closed level. (*B*) Channel open probability (P_o) was calculated at −80 mV from a total of nine paired experiments with WT CFTR before (*Control:* P_o = 0.246 ± 0.056) and after application of cyclosporin A (+*Cyclosporin A:* P_o = 0.233 ± 0.052), and upon subsequent addition of cyclophilin A (+*CsA*+*Cyclophilin A:* P_o = 0.234 ± 0.044).

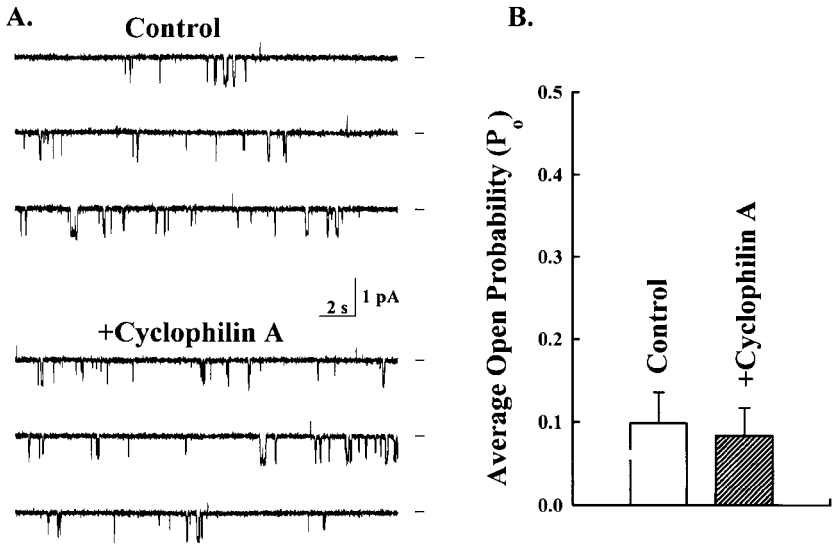

FIGURE 3 Lack of effect of cyclophilin A on ΔR(708–835) CFTR channel. (*A*) Single channel current traces are plotted for ΔR(708–835) CFTR channel at −80 mV test potential before (*Control*) and after the application of cyclophilin A (+*Cyclophilin A*). The records were obtained with 2 mM ATP and no PKA present in the cytosolic solution. The channel closed level was marked by the short bar on the right side of each current trace. (*B*) P_o was calculated at −80 mV from a total of four single channel experiments with ΔR(708–835) CFTR. The averaged P_o was 0.099 ± 0.037 (*Control*), and 0.084 ± 0.034 (+*Cyclophilin A*).

resulting mutant named P3A CFTR. The P3A CFTR mutant was incorporated into the planar lipid bilayer, and its function was compared with that of the wild-type CFTR. Similar to the WT CFTR channel, opening of the P3A CFTR channel strictly requires the presence of both ATP and PKA in the intracellular solution (Fig. 4 *A*, *right*). Without PKA, ATP alone is insufficient for opening the P3A channel (Fig. 4 *A*, *left*, $n = 5$), and without ATP, PKA alone could not induce opening of the P3A channel (Fig. 4 *A*, *middle*, $n = 3$). Opening the P3A CFTR channel, like WT CFTR, could be completely blocked by 3 mM DPC added to the extracellular solution (Fig. 4 *A*, *right*). The current-voltage relationship of the P3A CFTR channel is similar to that of the WT CFTR channel, with a slope conductance of 7.6 ± 0.3 pS and a reversal potential of 19.4 ± 3.2 mV under an asymmetric ionic condition of 200 mM KCl (intracellular)/50 mM KCl (extracellular) (Fig. 4 *B*). Thus, these mutations do not change the conduction properties of the CFTR channel.

However, the overall single channel activity is significantly increased for the P3A CFTR channel compared to WT CFTR. On average, activity of the P3A channel (P_o = 0.577 ± 0.090) was about twice that of WT CFTR channels measured at the same time (P_o = 0.204 ± 0.036) (Fig. 5, *A* and *B*; Fig. 8). This enhanced activity of the P3A CFTR channel was consistently observed in all the single channel experiments with five separate vesicle preparations. Thus, similar to the cyclophilin A-treated WT CFTR channel, the three proline-to-alanine mutations greatly increased the CFTR channel activity. Moreover, cyclophilin A does not further enhance the activity of the P3A CFTR channel (Fig. 5, *A* and *B*).

Open-time kinetic analysis of the P3A CFTR channel shows that, similar to the cyclophilin A-treated WT channel, the increased activity of the P3A CFTR channel is mainly due to the increase in the number of open events, not due to changes in the mean open lifetime of the channel (Fig. 5 *C*; Fig. 8). Like the WT CFTR and its cyclophilin A-treated channels, the P3A channel has two open states with mean open lifetimes of τ_{o1} = 56.6 ± 12.85 ms and τ_{o2} = 151.1 ± 35.5 ms, respectively (Fig. 5 *C*).

Fig. 6 shows the closed-time kinetic analysis with WT control, cyclophilin A-treated WT, and P3A CFTR channels. The channels under three circumstances contain a fast component in addition to several slow components. The fast component (τ_{c1}), representing the fast closing events within an open burst of the CFTR channel (Carson et al., 1995), has a time constant of ~20 ms, which is similar in WT control, WT treated with cyclophilin A, and P3A CFTR channels (Fig. 6 *A*). To facilitate identification of the slow closing components of the CFTR channels, a delimiter of τ_c = 40 ms was set to construct the closed-burst duration histograms (Fig. 6 *B*). The 40 ms represent the nadir between the fast and slow components of the histograms shown in Fig. 6 *B*. The closed-burst duration histograms of all three channels, WT, WT treated with cyclophilin A, and P3A CFTR, can be fitted with two exponential components. The intermediate closed state (τ_{c2}) has a time constant of 150–300 ms, and the long closed state (τ_{c3}) has a time constant of 1–2 s (Fig. 6 *B*). Compared to the WT CFTR, both cyclophilin A-treated WT and P3A CFTR channels have a shorter τ_{c2} (notice the left shift of the main peak of the histogram in Fig. 6 *B*). For the cyclophilin A-treated channel, the long closed time constant τ_{c3} is also shorter (~1 s) than that of the WT control (~2 s, Fig. 6 *B*). For the P3A CFTR channel, although τ_{c3} is not significantly different from that of the WT, the occurrence of τ_{c3} is significantly lower in P3A than in WT CFTR channels. The probability of the τ_{c3} is 12.4% of the closed-burst events for the P3A CFTR, compared to 25% of the closed-burst events for the WT CFTR channel (Fig. 6 *B, top and bottom panels*).

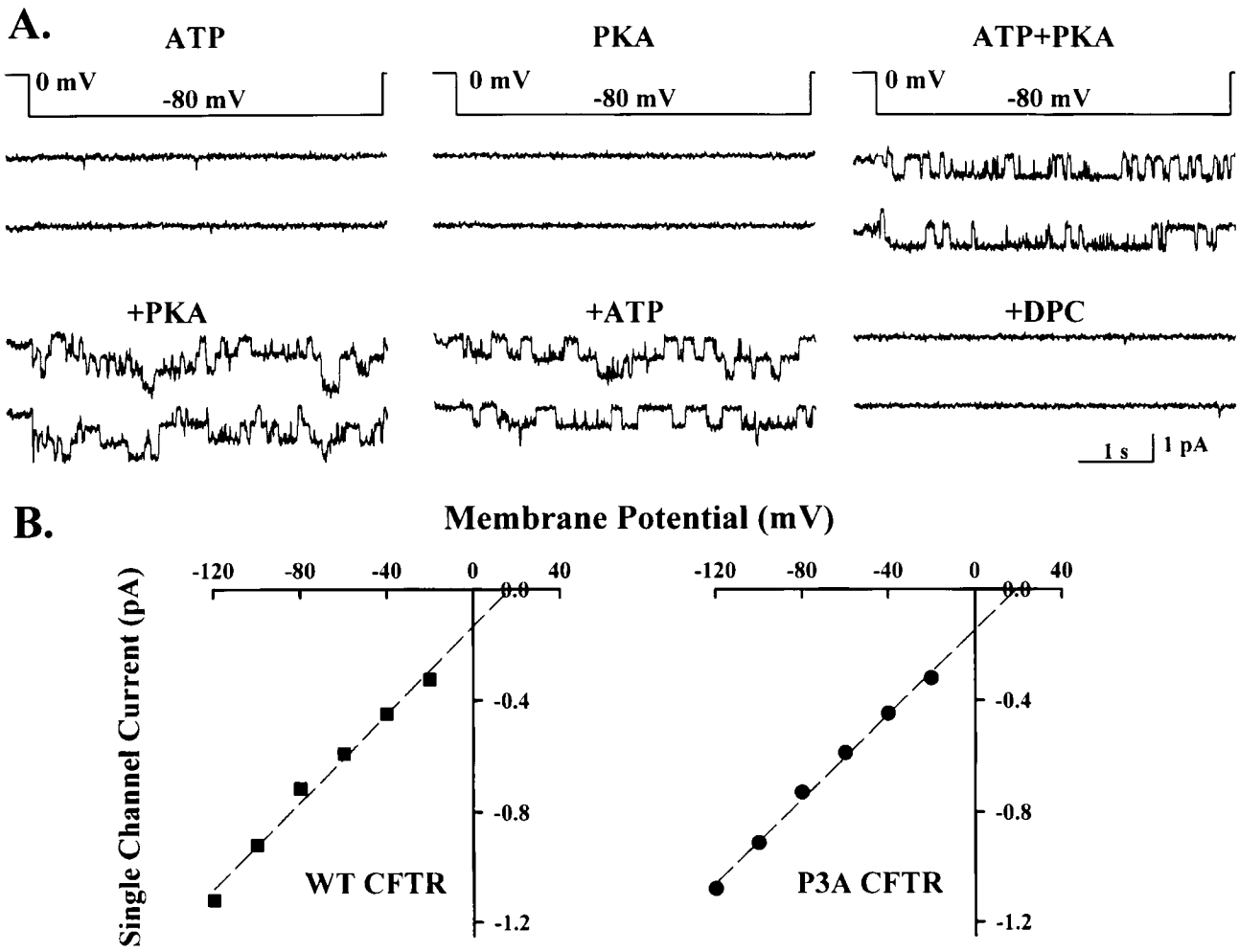

FIGURE 4 ATP- and PKA-dependent gating of the P3A CFTR channel. (*A*) P3A CFTR forms functional chloride channels in the lipid bilayer membrane. The channel opening absolutely requires the presence of both ATP and PKA in the *cis*-intracellular solution. ATP (2 mM, *left panel*), or PKA (50 units/ml, *middle panel*) alone is insufficient to induce channel opening. The channel activity was sensitive to block by 3 mM DPC added to the extracellular solution (*right panel*). (*B*) The current-voltage curves of the WT and P3A CFTR channel are plotted in the left and right panels, respectively. The dotted lines represent the best fit according to a linear equation: $I = G(V - V_{rev})$, with slope conductance of $G = 7.9 \pm 0.4$ pS and reversal potential of $V_{rev} = 17.0 \pm 5.3$ mV for WT CFTR; and $G = 7.6 \pm 0.3$ pS and $V_{rev} = 19.4 \pm 3.2$ mV for P3A CFTR.

Thus, similar to the cyclophilin A-treated WT CFTR channel, the proline-to-alanine mutations led to an increase in the opening rate of the CFTR channel without changes in the closing rate of the channel. Together, these results indicate that combined mutation of the three conserved proline residues leads to a PKA- and ATP-regulated chloride channel, with significantly higher activity than that of the WT CFTR channel, and with kinetic properties similar to the cyclophilin A-treated WT CFTR channel.

All three proline mutations are involved in the enhanced activity of the P3A CFTR channel

To investigate which proline is critical for the increased open probability of the P3A channel, we constructed all three single proline mutants (P740A, P750A, P759A) and incorporated each of them into the planar lipid bilayer. Fig. 7 *A* shows representative single channel current traces of the three single proline mutants recorded at −80 mV. Mutants P740A and P759A CFTR have similar open probability to WT CFTR, whereas P750A CFTR has significantly increased P_o. Data from multiple experiments for both wild-type and proline mutants are summarized in Fig. 8. The top panel shows the mean open probability (P_o) and the bottom panel shows the arithmetic mean open life time (τ_{mean}). Although the P_o of P3A CFTR is more than double that of the WT CFTR, its mean open life time is not significantly different from that of the wild type, indicating that P3A increases the P_o of CFTR mainly by increasing the number of channel open events. P740A CFTR had P_o similar to the wild type, while its mean open life time is significantly ($p < 0.05$) lower (closing faster) than that of the WT CFTR, so

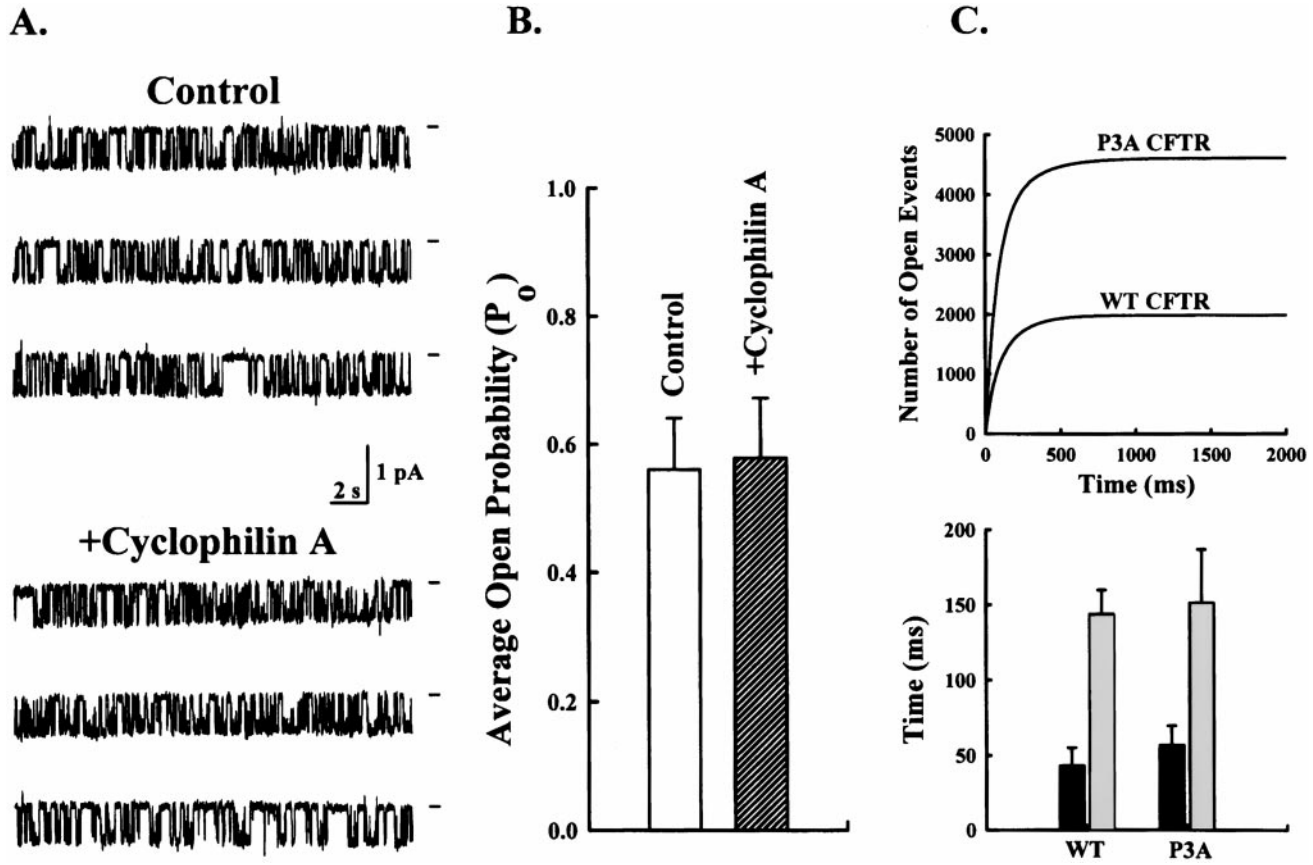

FIGURE 5 Cyclophilin A on P3A CFTR channel and P3A channel open-time histograms. (*A*) Single channel current traces are plotted for P3A CFTR channel at −80 mV holding potential before (*Control*) and after the application of cyclophilin A (+*Cyclophilin A*). The short bar on the right side of each current trace indicates the channel closed level. (*B*) P_o was calculated at −80 mV from a total of five paired experiments with P3A CFTR. The averaged P_o was 0.560 ± 0.081 (*Control*), 0.578 ± 0.094 (+*Cyclophilin A*). (*C*) Cumulative open-time histograms at −80 mV (*top panel*). Open events were analyzed with single channel currents recorded at 40 Hz cutoff filtering frequency. A total of 18-min channel recordings at −80 mV were used for both the WT ($n = 6$ bilayer experiments) and P3A CFTR ($n = 8$) channels. A total of 1891 and 4621 open events were detected, respectively, for the WT and P3A CFTR channels. The cumulative open-time histogram from individual experiments was fitted with the following equation: $y = W_{01}(1 - \exp(-t/\tau_{01})) + W_{02}(1 - \exp(-t/\tau_{02}))$, and the individual brief (τ_{01}) and long (τ_{02}) open-time constants were averaged, for the statistical kinetic analysis of the P3A CFTR channel (*bottom panel*). The averaged time constants τ_{01} (*black bar*) and τ_{02} (*gray bar*) were plotted (P3A: $\tau_{01} = 56.6 \pm 12.85$ ms, $\tau_{02} = 151.1 \pm 35.5$, $n = 8$) and compared with that of the WT CFTR (WT: $\tau_{01} = 42.9 \pm 15.0$ ms, $\tau_{02} = 143.7 \pm 16.0$, $n = 10$).

the P740A mutant must have a higher opening rate. This mutation could contribute to P3A channel function by increasing the number of channel openings. The mean open lifetime of P750A is not different from that of WT CFTR, but open probability of P750A is significantly higher than wild-type CFTR, though still significantly ($p = 0.044$) lower than P3A. Thus, P750A alone does not entirely account for the P3A channel activity, and the other two prolines also contribute to the high P_o of P3A CFTR. P759A CFTR shows no increase in open probability compared to the wild type, but its mean open life time is significantly longer than that of the wild-type CFTR.

To test whether mutation of prolines 750 and 759, but not proline 740, would have even higher P_o than P3A by combining the increased channel opening of P750A with the increased open life time of P759A, the double proline mutant P2A CFTR (P750A/P759A) was made. Representative channel current traces of P2A CFTR are plotted in Fig. 7 *B*, and summary data from multiple experiments are shown in Fig. 8. P2A has an increased mean open life time, like the P759A mutant, but its P_o is not significant higher than wild-type CFTR ($p = 0.46$). Thus, no single proline mutation, nor the double mutant combining the two most favorable single proline mutants, has P_o as high as P3A CFTR, and all three proline residues in the R domain of CFTR contribute to the optimal channel function of P3A CFTR.

Cyclophilin A fails to stimulate the activity of the ΔNEG2 CFTR channel

To further examine how the CFTR channel is stimulated through the isomerization around the proline residues within the R domain, we tested the effect of cyclophilin A on

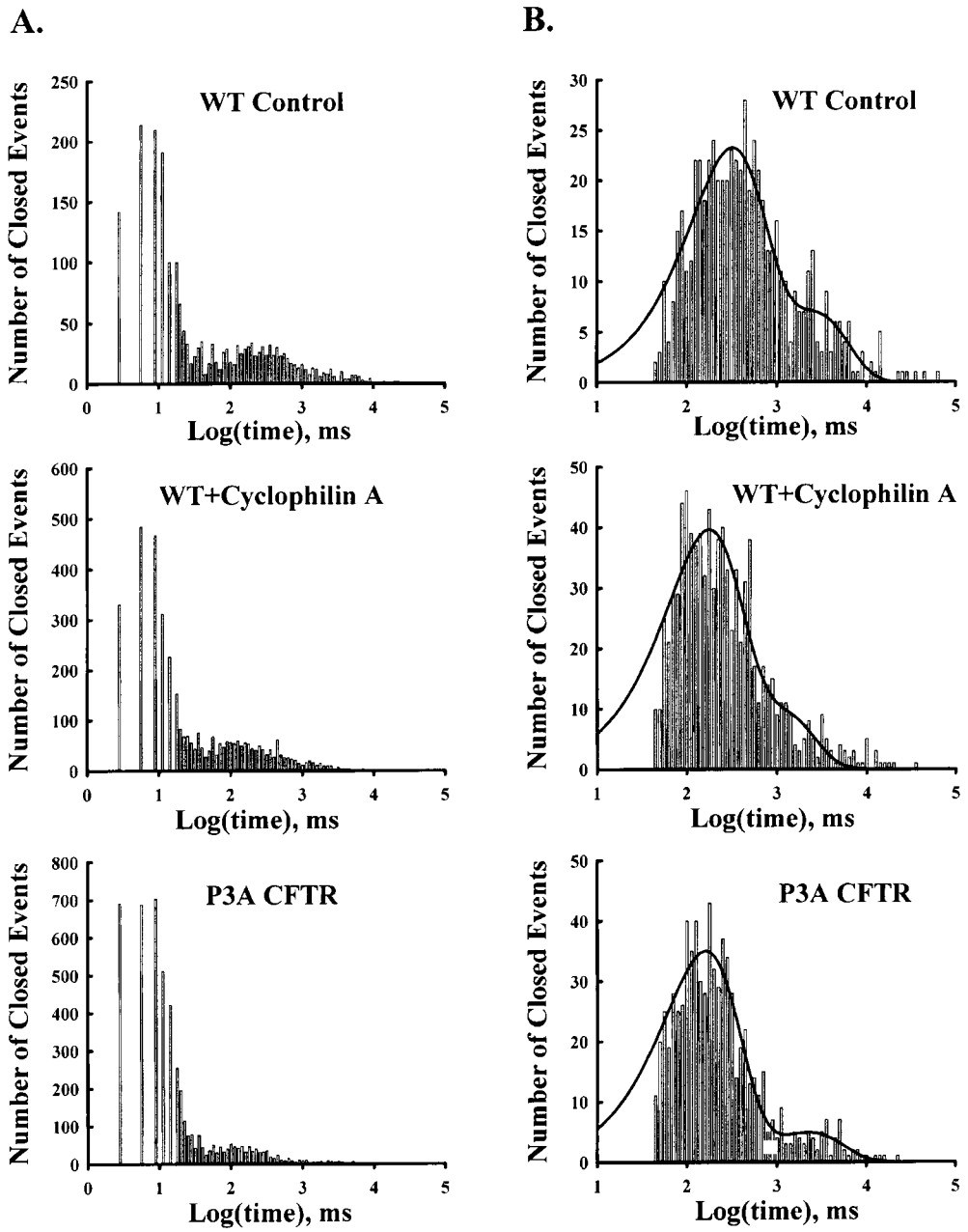

FIGURE 6 Closed-time histograms of the P3A and WT CFTR channels. (*A*) Closed-time histograms at −80 mV. Closed events were analyzed with single channel currents recorded at 40 Hz cutoff filtering frequency. A total of 18-min channel recordings at −80 mV were used for both the WT ($n = 6$ paired bilayer experiments) and P3A CFTR ($n = 8$) channels. (*B*) Close-burst histogram at −80 mV. Only the closing events longer than 40 ms were plotted using the same set of data files as in *A*. The histograms were fitted with the following equation: $y = N^*P_{c2}^* \exp((t - \alpha_2)/T - \exp((t - \alpha_2)/T)) + N^*P_{c3}^*\exp((t - \alpha_3)/T - \exp((t - \alpha_3)/T))$, Where N = the total number of closed events, P_{c2} = the probability of the intermediate closed component, and P_{c3} = probability of the long closed component, $T = \log(e)$ (where e is the natural exponential constant), $\alpha_2 = \log \tau_{c2}$, $\alpha_3 = \log \tau_{c3}$. The best-fit parameters were $N = 77$, $P_{c2} = 0.75$, $\tau_{c2} = 298.6$ ms, $P_{c3} = 0.25$, $\tau_{c3} = 2489.2$ ms for WT CFTR control; $N = 124$, $P_{c2} = 0.788$, $\tau_{c2} = 163.9$ ms, $P_{c3} = 0.212$, $\tau_{c3} = 1013.4$ ms for WT + Cyclophilin A; $N = 107$, $P_{c2} = 0.876$, $\tau_{c2} = 151.11$ ms, $P_{c3} = 0.124$, $\tau_{c3} = 2221.0$ ms for P3A CFTR.

another CFTR deletion mutant, ΔNEG2 CFTR, in which a short segment (a.a. 817–838) containing many negatively charged amino acids, but no proline, in the R domain was deleted. Like the ΔR CFTR channel, ΔNEG2 CFTR opens without PKA phosphorylation and is independent of PKA regulation (Adams et al., 1998). The ΔNEG2 (817–838) sequence, a 22-amino acid peptide, provided exogenously, stimulates the WT CFTR channel (Adams et al., 1998). We speculate that if the mechanism of action of proline isomerization is to present this critical sequence in the R domain

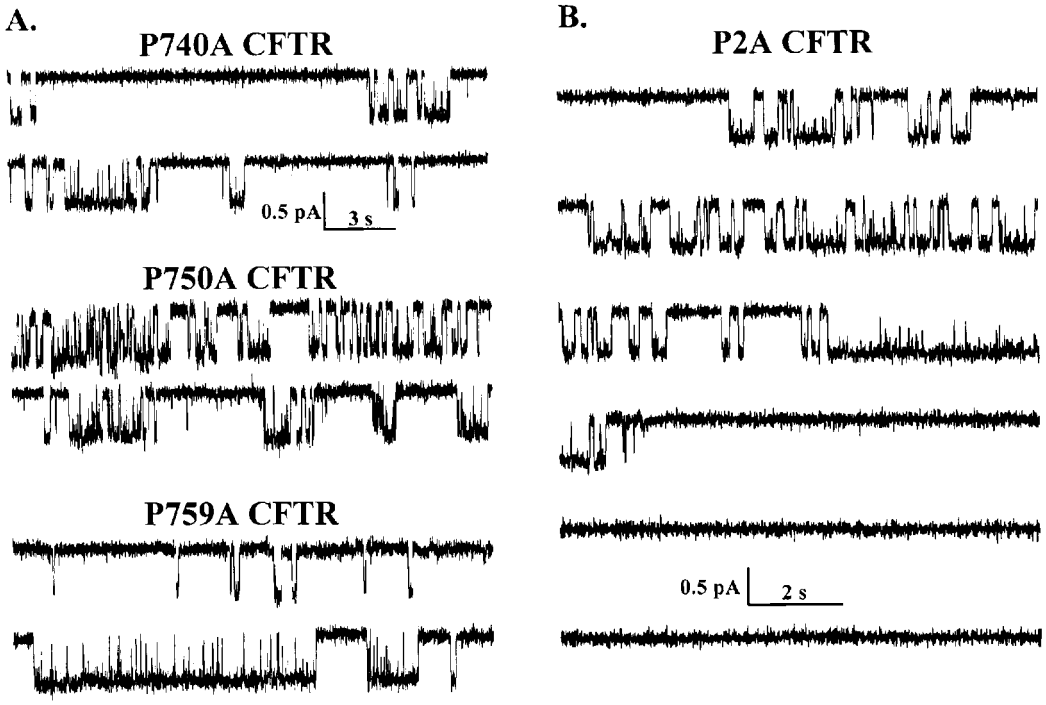

FIGURE 7 Reconstitution of CFTR single and double proline mutants. (*A*) Single channel current recordings for three CFTR single proline mutants are shown. Records are continuous 40-s current recordings at holding potential of −80 mV. (*B*) Representative single channel currents through a double proline mutant (P2A: P750A/P759A) are plotted.

more favorably for stimulation of channel openings, a mutant channel lacking the critical sequence should not respond to cyclophilin A.

Fig. 9 *A* shows representative single channel current traces of the ΔNEG2 CFTR mutants recorded at −80 mV. Upon the addition of cyclophilin A, the activity of the channel remains essentially unchanged. Regardless of the phosphorylation status of the channel, the average channel open probability was the same ($p = 0.74$) with and without cyclophilin A (Fig. 9 *B*). Thus, without the putative stimulatory sequence in the R domain, isomerization of the three conserved proline residues in the R domain has no effect on the channel activity.

The proline mutants of CFTR are fully processed in 293 HEK cells

To determine whether the proline mutants are processed normally in mammalian cells, they were introduced into the 293 HEK cells using the liposome-mediated gene transfection method (Xie et al., 1995, 1996) and their expression was confirmed by immunoprecipitation/Western blot assay. Cells transfected with WT CFTR expressed a fully glycosylated ~170 kDa CFTR protein (Fig. 10, *lane 2*). This blot (and many others) shows that cells transfected with P3A CFTR expressed comparable amounts of fully glycosylated CFTR protein (Fig. 10, *lane 3*), indicating the processing of P3A CFTR is similar to that of the WT CFTR in 293 HEK cells. The double (P2A; Fig. 10, *lane 4*) or single proline mutants (P740A, P750A, P759A; Fig. 10, *lanes 5–7*, respectively) were also fully glycosylated to a similar extent.

P3A supports chloride transport in live cells in excess of wild-type CFTR

To test whether the P3A CFTR supports chloride transport at the cell surface, an SPQ assay was performed on HEK 293 cells transfected with pCEP4(P3A) using WT CFTR-expressing cells and untransfected cells as controls (Fig. 11). Untransfected 293 HEK cells exhibited negligible forskolin-induced chloride transport activity, whereas cells expressing the WT CFTR exhibited significant increase in the rate of chloride efflux upon forskolin stimulation. In cells transfected with P3A CFTR, the basal chloride efflux rate was similar to that of untransfected cells and cells transfected with WT CFTR in the absence of forskolin stimulation. Upon forskolin stimulation, cells expressing P3A CFTR exhibited a large increase in the rate of chloride efflux, which is significantly greater ($p = 0.001$) than that seen with WT CFTR. Thus, P3A CFTR can support chloride transport across the surface membrane of 293 HEK cells, and furthermore, activation of the P3A CFTR channel appears to be tightly controlled by the cAMP-dependent

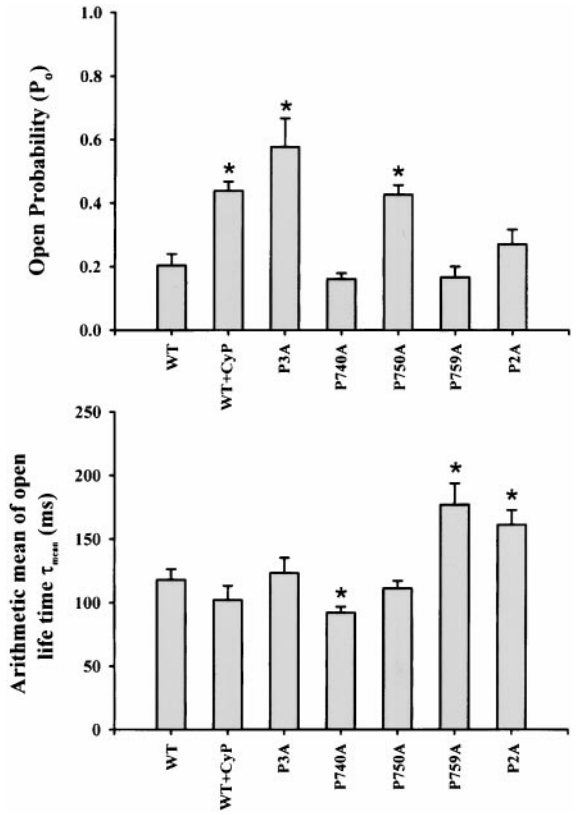

FIGURE 8 Open probability and mean open life time of WT and CFTR proline mutants. *Top:* single channel open probabilities (P_o) were calculated at −80 mV for both wild-type and each CFTR proline mutant. The data for WT, cyclophilin A-treated WT, and P3A mutant are calculated from more than five separate experiments recorded during the time period when single proline mutants were also studied. The averaged values were $P_o = 0.204 \pm 0.036$ (WT), 0.438 ± 0.029 (WT + CyP (Cyclophilin A)), 0.577 ± 0.090 (P3A CFTR), 0.161 ± 0.018 (P740A), 0.426 ± 0.030 (P750A), 0.166 ± 0.034 (P759A), 0.272 ± 0.059 (P2A), respectively. Asterisks indicate P_o is significantly different from wild type. *Bottom:* the mean open lifetimes for wild-type and each proline mutant were calculated from the same set of experiments as above. Asterisks indicate significant difference from wild type.

protein kinase pathway, as was observed in the single channel studies (Fig. 4).

Although the P_o of P3A CFTR is more than twice that of WT CFTR, the average chloride efflux rate of P3A CFTR in cells is only 1.4 times that of WT CFTR (Fig. 11). This phenomenon may occur because in 293 HEK cells (which express the Epstein-Barr Nuclear Antigen (EBNA)), very high transfection efficiency and copy numbers can be achieved with the pCEP4 vector, which contains the ori P sequences to promote replication soon after the plasmid enters the cell. In another system, cells transfected with increasing doses of WT CFTR display maximal chloride transport at lower-than-maximal expression of CFTR mRNA or protein (Rosenfeld et al., 1994). Expression of chloride transport may be near-maximal in 293 HEK cells even with wild-type CFTR. Thus this system may underestimate the difference in the relative channel activity.

DISCUSSION

When the R domain is phosphorylated a negative charge is added and a conformational change ensues; this allows ATP binding and hydrolysis at the nucleotide binding domains, and with transduction of this change to the pore the CFTR channel opens. We have taken the advantage of cyclophilin A, a proline isomerase, to demonstrate that changes in conformation contribute to further channel opening after phosphorylation has allowed initial opening of CFTR channel. Our studies focus on three highly conserved proline residues (conserved among human, cow, rabbit, mouse, *Xenopus*, and squalus), at positions 740, 750, and 759 in the R domain, in the midst of consensus PKA phosphorylation sites (at residues 737 and 795), which appear to account for the results we observed.

CFTR captured in the planar lipid bilayer responds to cyclophilin A by doubling the channel open probability. This effect is blocked and reversed by cyclosporin A. Since cyclophilin A had no effect on P_o of the ΔR(708−835) CFTR channel, we speculated that prolines in the deleted segment might be the targets of cyclophilin A isomerization. Three prolines in the deleted segment (P740, P750, and P759) are highly conserved across species, more so than the other six prolines in the R domain, three of which are not in good consensus sequences for the cyclophilin enzyme. When the prolines at 740, 750, and 759 are mutated to alanines, stimulation by cyclophilin A is eliminated, suggesting that the other prolines do not contribute to the activation by cyclophilin A. In addition, the open probability of this P3A mutant is significantly increased compared to the wild type, and the kinetic properties of the mutant are similar to those of cyclophilin A-treated WT CFTR. These data support the hypothesis that cyclophilin A acts through isomerization about peptidyl-prolyl bonds at these three proline residues, and the resulting change in conformation favors channel openings.

Cyclophilin A is a very efficient *cis-trans* peptidyl-prolyl isomerase when tested with a synthetic substrate N-carboxypropionyl-Ala-Xaa-Pro-Phe-*p*-nitroanilide. It is effective when Xaa is Gly, Ala, Val, Leu, Phe, His, Lys or Glu, but more hydrophobic amino acids are preferred (Harrison and Stein, 1990). The sequences surrounding prolines 740 (Val-Pro), 750 (Leu-Pro), or 759 (Gly-Pro) should be preferred substrates for the enzyme. Cyclophilin A should, therefore, favor the *trans* configurations about these bonds. Another way to favor the *trans* configuration is to substitute another amino acid, such as alanine, for proline, which tends to favor the *cis*-peptidyl conformation. In the mutational analysis, substitution of all three prolines is required for maximal channel activity. The properties of the P750A mutant are most similar to cyclophilin A-treated WT CFTR

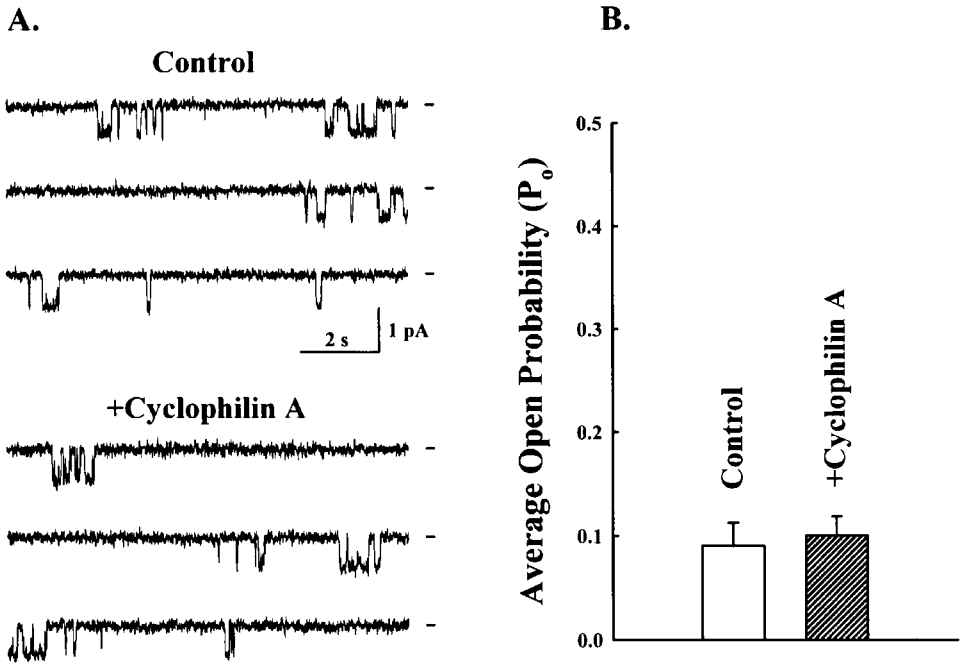

FIGURE 9 Lack of effect of cyclophilin A on ΔNEG2 CFTR channel. (*A*) Single channel current traces are plotted for ΔNEG2 CFTR channel at −80 mV test potential before (*Control*) and after the application of cyclophilin A (+*Cyclophilin A*). The records were obtained with 2 mM ATP and no PKA present in the cytosolic solution. The channel closed level was marked by the short bar on the right side of each current trace. (*B*) P_o was calculated at −80 mV from a total of six paired bilayer experiments with ΔNEG2 CFTR. The averaged P_o was 0.090 ± 0.022 (*Control*), and 0.101 ± 0.018 (+*Cyclophilin A*).

or the triple mutant P3A, in that P_o is increased due to the increased number of channel openings and not increased channel open time. However, a further increase in P_o is achieved by mutation of prolines 740 and 759, which by themselves do not result in increased P_o. Thus, favoring all *trans*-peptidyl bond configurations at these site results in a conformational change that optimally stimulates the channel opening with phosphorylation, while leaving intact the ability of the channel to remain in the closed state without phosphorylation. Because stimulation by cyclophilin A is observed if the enzyme is added after the channel has been phosphorylated and subsequent inhibition of cyclophilin A activity by cyclosporin A can result in reversal of the stimulation (data not shown), it is unlikely that cyclophilin A acts simply by presenting the phosphorylation sites in a particularly favorable order (Csanady et al., 1998). It is also

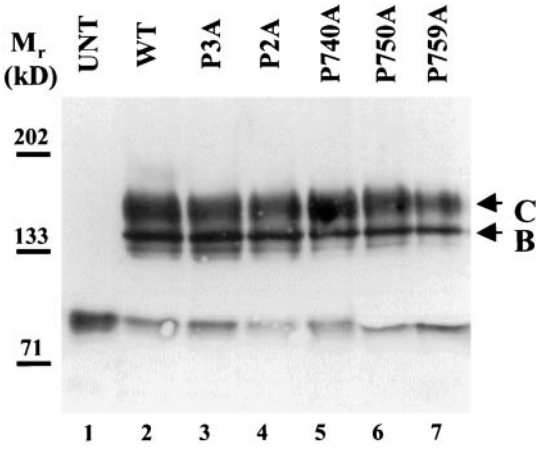

FIGURE 10 Heterologous expression of wild-type CFTR and proline mutants in 293 HEK cells: 293 HEK cells transfected with pCEP4(WT), pCEP4(P3A), pCEP4(P2A), pCEP4(P740A), pCEP4(P750A), or pCEP4(P759A) were immunoprecipitated and blotted as described in the Materials and Methods; mAb24-1 that recognizes the C-terminus of CFTR was used in the immunoprecipitation/Western blot. Arrows labeled *B* and *C* indicate CFTR band B (core glycosylated) and band C (mature glycosylation). *Lane 1 (UNT):* untransfected 293 HEK cells; *lane 2:* WT CFTR expressing cells; *lanes 3–7:* P3A, P2A, P740A, P750A, and P759A CFTR expressing cells, respectively.

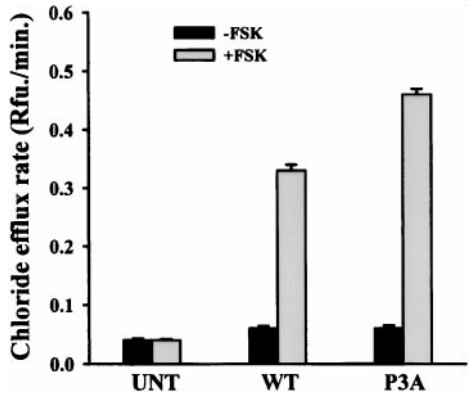

FIGURE 11 SPQ assay of chloride transport in 293 HEK cells transfected with P3A CFTR. Chloride efflux rate was calculated as described previously (Xie et al., 1995) and plotted as the relative fluorescence unit change per minute (Rfu/min.). Black bars indicate the rate of basal chloride efflux (*UNT:* 0.04 ± 0.003, 98 cells in 3 experiments; *WT*: 0.06 ± 0.004, 84 cells in 4 experiments; *P3A:* 0.06 ± 0.005, 80 cells in 4 experiments) and gray bars indicate the rate of chloride efflux upon forskolin (10 μM) stimulation (*UNT:* 0.04 ± 0.002; *WT*: 0.33 ± 0.01; *P3A:* 0.46 ± 0.01). *UNT:* untransfected cells.

unlikely that these reagents inhibit phosphatases to stimulate channel openings, as our prior data indicate that the bilayer system and vesicles prepared from HEK 293 cells are free of significant phosphatase activity (Ma et al., 1997). Channels in our system show little rundown, even over 30–45 min in the presence of protein kinase A inhibitors, and activity is not enhanced by phosphatase inhibitors. Rather, it is likely that isomerization about the peptidyl-prolyl bond changes the conformation of the phosphorylated R domain, and this conformational change, above and beyond the changes in charge and conformation induced by phosphorylation, enhances the stimulatory function of the R domain.

The R domain of CFTR, when unphosphorylated, functions in an inhibitory capacity. The evidence for this is that when WT CFTR is not phosphorylated, it can not open even at very high ATP concentrations, but deleting a portion of the R domain allows the channel to open without phosphorylation. Moreover, adding back unphosphorylated R domain to the WT CFTR channel captured in the planar lipid bilayer results in channel closure. This inhibitory function of the R domain is not altered by cyclophilin A or by mutation of the critical prolines (P3A), since in neither case does the channel open without phosphorylation. However, the R domain also functions in a stimulatory capacity when it is phosphorylated. When phosphorylated R domain is added back to the CFTR ΔR channel captured in the planar lipid bilayer or patch clamp, channel openings are stimulated and open probability increases (Ma et al., 1997; Winter and Welsh, 1997). Recently, a 22-amino acid segment of the R domain, which lacks phosphorylation sites but is heavily negatively charged, has been reported to stimulate channel activity (Adams et al., 1998), also by increasing the number of channel openings. Similarly, in the P3A and cyclophilin A-treated WT CFTR channels, channel openings, not open time, increase. However, cyclophilin A is unable to stimulate the ΔNEG2 CFTR channel, even though all proline residues are retained. These results suggest that the stimulatory sequence of the R domain resides within the NEG2 region. Consistent with our data, Cotten and Welsh (1997) showed that covalent modification of C832 in the NEG2 region irreversibly stimulated CFTR channel activity. These data, taken together, suggest that the isomerization about the three critical proline residues promotes channel openings by improving the ability of the R domain to present its stimulatory sequences (contained in NEG2) to the appropriate site(s). As it is generally considered that ATP binding and hydrolysis at the first nucleotide binding domain (NBD1) opens the phosphorylated channel (Gadsby and Nairn, 1994; Carson et al., 1995; Ma and Davis, 1998), it is likely that the conformational change about the critical prolines promotes channel openings by improving the ability of the R domain to present its stimulatory sequences to the appropriate site within NBD1 of CFTR.

The P3A mutant has properties that may be favorable for gene therapy purposes. The P_o of this channel is greatly increased compared to wild-type CFTR, the mutant is processed normally and functions at the cell surfaces, yet activation of the mutant is under physiologic control. Thus, for every molecule of P3A CFTR expressed, it would be possible to achieve double the chloride flux of a molecule of WT CFTR. Therefore, a lower level of expression of exogenous gene could achieve the same level of chloride transport. Moreover, human epithelial cells transfected with P3A-CFTR display cAMP-stimulated chloride efflux significantly in excess of the wild type. Because the efficiency of gene transfer has been problematic in gene therapy experiments for cystic fibrosis (Grubb et al., 1994; Blomer et al., 1996; Verma and Somia, 1997), improvements in the per-molecule efficiency of the protein product might be useful. Although other CFTR mutants have chloride transport in excess of WT CFTR, they are either not processed efficiently (e.g., P574H or H949Y) (Sheppard et al., 1996a, b; Seibert et al., 1996) or open without PKA stimulation (e.g., CFTR-D836X), and thus are not subject to physiologic regulation (Sheppard et al., 1994).

By using cyclophilin A as a probe and following up with the mutants of highly conserved proline residues in the R domain, we have shown that *cis-trans* isomerization about peptidyl-prolyl bonds in the R domain increases the open probability of the CFTR channel, above and beyond the charge and conformational effects of phosphorylation. Activation occurs by increasing the number of channel openings, not by changes in channel open time, and we speculate that the conformational changes from isomerization present stimulatory sequence(s) in the R domain more effectively to their cognate site(s) within the CFTR molecule. Moreover, this effect may be exploitable for therapeutic purposes.

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