Function of the R Domain in the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel

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For a cystic fibrosis transmembrane conductance regulator (CFTR) channel to enter its open state, serine residues in the R domain must be phosphorylated by cAMP-dependent protein kinase, and intracellular ATP must bind to the nucleotide-binding folds and subsequently be hydrolyzed. CFTR with its R domain partially removed, ΔR(708–835)-CFTR, forms a chloride channel that opens independently of protein kinase A phosphorylation, with open probability approximately one-third that of the wild type CFTR channel. Deletion of this portion of the R domain from CFTR alters the response of the channel to 5'-adenyllylimidodiphosphate, pyrophosphate, and vanadate, compounds that prolong burst duration of the wild type CFTR channel but fail to do so in the ΔR-CFTR. In addition, the addition of exogenous unphosphorylated R domain protein, which blocks the wild type CFTR channel, has no effect on the ΔR-CFTR channel. However, when the exogenous R domain is phosphorylated, significant stimulation of the ΔR-CFTR channel results; \( P_o \) increases from 0.10 to 0.22. These data are consistent with a model for CFTR function in which the R domain in the unphosphorylated state interacts with the first nucleotide binding fold to inhibit either binding or hydrolysis of ATP or transduction of the effect to open the pore, but when the R domain is phosphorylated, it undergoes conformational change and interacts at a separate site in the first nucleotide binding fold to stimulate either binding or hydrolysis of ATP or transduction of the effect to open the pore.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of a family of ATP-binding cassette transporters between the predicted first nucleotide binding fold and the predicted second membrane-spanning domain (9, 11). The R domain is a special feature of the CFTR molecule, unique among members of the ATP-binding cassette transporter family. Phosphorylation of serine residues in the R domain is a prerequisite for opening of the CFTR channel. Only the phosphorylated CFTR channel can open in response to ATP binding and hydrolysis (5–8).

Rich et al. (9) demonstrated that deletion of 128 amino acids from the R domain of CFTR, ΔR(708–835), leads to a chloride channel that opens without PKA phosphorylation. This portion of the R domain corresponds to sequences that are not conserved in related proteins, i.e., MDR1 and STE6 (9–11). The portion of the R domain that remains in ΔR-CFTR corresponds to amino acids that are present in other ATP-binding cassette transporters between the predicted first nucleotide binding fold and the predicted second membrane-spanning domain (9, 11). Our studies show that unphosphorylated R domain protein synthesized in vitro (amino acids 590–858) interacts specifically with CFTR to inhibit chloride conductance in a phosphorylation-dependent manner (12). These studies were interpreted to be consistent with the hypothesis that the putative "gating particle" of the CFTR channel resides within the R domain.

The first and second NBF of CFTR share sequence similarity in certain conserved regions such as Walker A and Walker B motifs, but the overall amino acid homology between the two NBFs of CFTR is only ~30%. Functional studies of CFTR containing site-directed mutations in NBF1 and NBF2 suggest that the two NBFs have different roles in the gating of the CFTR channel (13–15). Mutations predicted to interfere with nucleotide hydrolysis in the first NBF reduce the channel opening rate, but the corresponding mutations in the second NBF result in prolonged channel openings (15). The use of compounds that alter the ATP hydrolysis cycle of CFTR, such as 5'-adenyllylimidodiphosphate (AMP-PNP), P\(_i\), and vanadate (VO\(_4^\text{-}\)), provided evidence that hydrolysis of ATP is not only required for channel opening but also is involved in channel closure from the bursting state (16–19). Based on these studies, it was proposed that ATP hydrolysis at NBF1 initiates a burst of activity and that hydrolysis at NBF2 terminates a burst of open events (15).

This dual regulatory mechanism of the CFTR channel (PKA phosphorylation of the R domain and ATP binding and hydrolysis by the NBFs) requires coordinated interactions among the three intracellular domains: NBF1, R, and NBF2. However, little is known about the functional consequences of such interaction. It has been suggested that the rate of ATP hydrolysis by the two NBFs is sensitive to the degree of R domain phosphorylation (16), but it is not clear how the phosphorylation-induced conformational change in the R domain is connected...
with the binding and hydrolysis of ATP by the NBFs, which in turn controls channel opening and closing transitions. To explore the potential interaction between the R domain and the NBFs in the function of the CFTR channel, we compared the single channel properties of the wild type CFTR and its R domain deletion mutant, ΔR(708–835)-CFTR. The wild type and AR-CFTR were expressed in HEK 293 cells, from which microsomal membrane vesicles were isolated for single channel studies using the bilayer reconstitution technique. Consistent with the early work by Rich et al. (9), we found that ΔR-CFTR formed a chloride channel that opens independently of PKA phosphorylation. The AR-CFTR channel had open probability approximately one-third that of the wild type CFTR channel, due to fewer, shorter openings, which could not be prolonged by agents that increase burst duration in the wild type channel: AMP-PNP, vanadate, and PPi. Although the pore properties of the ΔR-CFTR channel were comparable with wild type, the addition of exogenous unphosphorylated R domain did not reduce open probability ($P_o$), as it does for the wild type channel. This observation casts doubt upon the hypothesis that the exogenous R domain acts as a gating particle. However, phosphorylated R domain increased the opening rate, but not the exogenous R domain acts as a gating particle. However, the effect of R domain protein on the CFTR channel, 20 μl of either phosphorylated or unphosphorylated R domain protein was added to the cis (intracellular) chamber. Control was the rabbit reticulocyte lysate mixture with no translated protein or the lysate used to translate the proteins of the brome mosaic virus, one of which is of the same molecular weight as the R domain protein (12).

**Isolation of Microsomal Membrane Vesicles from HEK 293 Cells—** The HEK 293 cells transfected with pCEP4(CFTR) or pCEP4(ΔR(708–835)-CFTR) were harvested and homogenized using a combination of hypotonic lysis and Dounce homogenization in the presence of protease inhibitors (5 μM diisopropyl fluorophosphate, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 10 mg/ml benzamidine) (12, 21). Microsomal membrane vesicles were isolated after sequential centrifugation at 1,000 × g for 30,000 × g; resuspended in a buffer containing 250 mM sucrose, 10 mM HEPES-Tris (pH 7.2) at a protein concentration of 3–7 mg/ml, and stored at −75 °C until use. Reconstitution of CFTR Channel in Lipid Bilayer Membrane—Lipid bilayer membranes were formed across an aperture ~200 μm in diameter with a lipid mixture of phosphatidylethanolamine/phosphatidylserine/cholesterol (6:6:1). The lipids were dissolved in decane at a concentration of 20–240 mg/ml (12, 21). The recording solutions contained the following: cis (intracellular, 1-ml volume), 200 mM KC1, 2 mM ATP, 1 mM MgCl2, and 10 mM HEPES-Tris (pH 7.4); trans (extracellular, 3-ml volume), 50 mM KC1, 10 mM HEPES-Tris (pH 7.4). Unless otherwise indicated, recordings of a single CFTR channel with ΔR(708–835)-CFTR were always performed without PKA present in the recording solution, and those with wild type CFTR were always performed with a 50 unit/ml concentration of PKA catalytic subunit (Promega) present in the cis-solution. Because the agonists for CFTR channel (ATP or PKA) were only present in the cis-solution, this condition selected only CFTR channels that were oriented in the bilayer membrane in the cis-intracellular trans-extracellular manner.

The data presented in this study were obtained with four different preparations of microsomal membrane vesicles isolated from the HEK 293 cells transfected with ΔR(708–835)-CFTR cDNA. The total number of experiments was 130. For the wild type CFTR, the total number of experiments was larger than 400.

**Analysis of Single Channel Data—** Single channel currents were recorded with an Axopatch 200A patch clamp unit (Axon Instruments). Data acquisition and pulse generation were performed with a 486 computer and 1200 Digidata A/D-DA converter (Axon Instruments). The currents were filtered at a cut-off frequency of 100 Hz through an eight-pole Bessel filter and sampled at 2.5 ms/point. Single channel data analyses were performed with pClamp software (Axon Instruments).

**RESULTS**

Partial Deletion of R Domain from CFTR Leads to a Chloride Channel That Opens Independently of PKA—The HEK 293 cells transfected with wild type CFTR cDNA or ΔR(708–835)-CFTR cDNA under the control of cytomegalovirus promoter expressed a large amount of CFTR proteins, detected in a Western blot (Fig. 1A). When microsomal membrane vesicles...
isolated from these cells transiently expressing the wild type and mutant CFTR proteins were fused with the lipid bilayer membrane, functional chloride channels were routinely detected (Fig. 1B). The wild type CFTR formed a linear conductance chloride channel with slope conductance of $8.2 \pm 0.6 \text{ pS}$ in 200 mM KCl, the opening of which requires absolutely the presence of ATP (2 mM) and PKA (50 units/ml) in the cis-intracellular solution (12, 21, 22). The wild type CFTR channel exhibits slow kinetics of gating with mean open lifetimes of $\tau_{o1} = 11.8 \text{ ms}$ and $\tau_{o2} = 189.6 \text{ ms}$ (Fig. 2A) and an average $P_o$ of 0.364 ± 0.042 at −80 mV (Fig. 2B).

The ΔR(708–835)-CFTR also formed functional chloride channels in the lipid bilayer membrane (Fig. 1B). These channels were linear with slope conductance of $8.0 \pm 0.6 \text{ pS}$. Opening of the ΔR-CFTR channel still requires the hydrolysis of ATP, since AMP-PNP (0.5–4 mM) alone is insufficient to induce opening. Similar to the wild type CFTR, the ΔR-CFTR channel was sensitive to blockade by diphenyl carboxylate added to the extracellular solution (Fig. 1B). The ΔR-CFTR channel remained open, even in the absence of PKA phosphorylation, but with average $P_o$ in 2 mM intracellular ATP of $0.122 \pm 0.012$, about one-third that of the wild type channel (Fig. 2A).

To test whether PKA phosphorylation can enhance the activity of the ΔR-CFTR channel, experiments were performed with the same channel before and after the addition of PKA to the intracellular solution. As shown in Fig. 3, there were no changes in $P_o$ following the addition of PKA (50 unit/ml concentration of the catalytic subunit) in seven paired experiments. To test whether the ΔR-CFTR channel had no response to PKA because it was phosphorylated in vivo by tonic PKA activity, we treated the channel with protein phosphatase 2A, (Promega), which reduced $P_o$ of the fully phosphorylated wild type channel from over 0.31 to 0.02 within 6 min in seven paired experiments. There was no change in $P_o$ of ΔR-CFTR following protein phosphatase 2A treatment for over 20 min ($n = 4$). Thus, neither PKA phosphorylation nor PP2A dephosphorylation changed the $P_o$ of ΔR-CFTR. This channel was not regulated by phosphorylation.

Further single channel data analyses revealed different gating properties between the wild type and ΔR-CFTR channels (Fig. 2). First, the transition rate into the open state was reduced in ΔR-CFTR, since the number of open events was significantly less (compare the plateau phase of the cumulative open time histograms in Fig. 2B). Second, the distribution of open lifetimes was different (Fig. 2B). The cumulative open time histogram of the wild type CFTR channel could be fitted with two exponential functions with time constants of 12 and 190 ms, whereas the ΔR-CFTR channel could only be fitted with one exponential function with a time constant of 78 ms. Thus, it appears that the wild type CFTR channel contained two open states and that the ΔR-CFTR channel contained only one open state, which can be resolved at a time resolution of 60 Hz cut-off frequency.

Thus, the ΔR-CFTR channel is not regulated by PKA phosphorylation, although a phosphorylation site known to be used in wild type CFTR, S660, remains intact in ΔR-CFTR. Since the $P_o$ of ΔR-CFTR channel is low even in the presence of PKA, we postulated that the decreased opening rate of the ΔR-CFTR channel (Fig. 2B) is due at least in part to alterations in the ATP-dependent gating of the CFTR channel.

AMP-PNP Does Not Stabilize the Open State of the ΔR-CFTR Channel—AMP-PNP, a nonhydrolyzable analogue of ATP, stabilizes the open state of the phosphorylated wild type CFTR channel in the presence of ATP (16, 17, 19, 24). In the presence of 2 mM ATP, AMP-PNP (0.4–5 mM) induced long open states in the wild type CFTR channel expressed in HEK 293 cells (Fig. 4A), with an increase in mean open lifetime from 96.0 ± 9.3 ms to 133.2 ± 15.6 ms (Fig. 4B). In contrast, AMP-PNP (2 mM) had no effect on either the open lifetime or the $P_o$ of the ΔR-CFTR channel (Fig. 4B). The effect of AMP-PNP on ΔR-CFTR was independent of PKA phosphorylation (data not shown). Higher concentrations of AMP-PNP (>10 mM) inhibited the opening rate of both wild type and ΔR-CFTR channels (data not shown).

PP, Fails to Prolong the Open State of the ΔR-CFTR Channel—We next tested the effect of PP on the wild type and ΔR-CFTR channel. PP, had a biphasic effect on the wild type CFTR channel (Fig. 5). At 0.2–5 mM, PP, increased $P_o$ of wild type CFTR by promoting long open states of the channel (Fig. 5, left panels), but at concentrations >10 mM, PP, reduced $P_o$. In contrast, the stimulating effect of PP, was not seen in the ΔR-CFTR channel (Fig. 5, right panels). In more than 14 experiments, we never observed any increase in $P_o$ or burst duration of the ΔR-CFTR channel following the addition of PP, (0.2–10 mM). Rather, $P_o$, even at low concentrations, consistently inhibited the opening rate of the ΔR-CFTR channel (Fig. 5B). At very high concentrations of PP, both the CFTR and the ΔR-CFTR channels opened predominantly to the 3-pS conductance state, which complicated calculation of $P_o$. 

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**FIG. 1. Expression of wild type and ΔR(708–835)-CFTR in HEK 293 cells.** pCEP4 eukaryotic expression vector containing the CFTR deletion mutant, ΔR(708–835), was generated by replacing the corresponding restriction fragment (BspI → PmlI) in pCEP4(WT-CFTR) with that from the pBlueScript(ΔR(708–835)-CFTR). A. Western blots of HEK 293 cells transfected with pCEP4(CFTR) or pCEP4(ΔR(708–835)). Two monoclonal antibodies, mAb13–1, which recognizes the R domain, and mAb24–1, which recognizes the C terminus of CFTR, were used in the immunoprecipitation/Western blot procedure. mAb13-1 and mAb24-1 recognized mature fully glycosylated CFTR protein of 170 kDa in cells transfected with pCEP4(CFTR). ΔR(708–835)-CFTR lacks the epitope for mAb13-1; thus, it could only be detected with mAb24–1. B. Selected single channel currents from a ΔR(708–835)-CFTR channel incorporated into the lipid bilayer membrane. Traces were acquired at a holding potential of −80 mV and obtained from a continuous experiment. The recording solution contained 200 mM KCl (cis-intracellular) and 50 mM KCl (trans-extracellular). The cis-solution contained 2 mM ATP with no PKA present. The concentration of diphenyl carboxylate was 3 mM.
It has been observed in several laboratories that the ability of CFTR to be locked open by PPi and ATP analogs depends on the opening rate of the channel (16, 25). We also observed that the effect of PPi and AMP-PNP on the wild type CFTR correlated with \( P_o \) of the channel; the lock-open effect is faster with a higher \( P_o \) channel (data not shown). If the long open states were just a statistical probability that occurred with enough openings, then observing the \( \Delta R \)-CFTR channel, with \( P_o \) about one-third that of the wild type channel, for a longer time period should eventually allow us to observe prolonged open states. We continued recording of the \( \Delta R \)-CFTR channel in typical experiments either with or without PKA for 10 min without ever observing prolonged channel openings. After the addition of AMP-PNP (2 mM, \( n = 6 \)) or PPi (0.2–5 mM, \( n = 17 \)), we recorded the channel for over 20 min and never observed the prolonged open state. The open time histogram demonstrating this for PPi is displayed in Fig. 10. We conclude that the failure of the \( \Delta R \)-CFTR channel to enter the bursting state, with or without these drugs, is not merely the consequence of low \( P_o \) and fewer openings of the channel but represents a distinct functional difference between wild type CFTR and \( \Delta R \)-CFTR.

**VO\(_4^−\) Inhibits \( P_o \) of the \( \Delta R \)-CFTR Channel**—Vanadate, an analog of P\(_i\), inhibits a variety of transport ATPases (26). It binds tightly at a site from which P\(_i\) is released following its cleavage from ATP and thus precludes further cycles of ATP hydrolysis. Vanadate enhances the activity of the native CFTR channel in the heart membrane (17) and the wild type CFTR channel heterologously expressed in different cell lines (19, 24). The effect of VO\(_4^−\) on the wild type and \( \Delta R \)-CFTR channels is shown in Fig. 6. Single channel records indicate that the stimulatory effect of VO\(_4^−\) on the wild type CFTR channel is largely due to the increase in open lifetime. At VO\(_4^−\) concentrations > 1 mM, the wild type CFTR channel remained open essentially all of the time (\( n = 14 \), Fig. 6, left). In contrast, VO\(_4^−\) reduced \( P_o \) of the \( \Delta R \)-CFTR channel (\( n = 6 \); Fig. 6, right).

**Subconductance States of the Wild Type and \( \Delta R \)-CFTR Channels**—We have shown previously that the wild type CFTR channel contained two distinct subconductance states of \textasciitilde -5–6 pS (M) and \textasciitilde -3 pS (L) in addition to the full conductance state (8 pS, H) (21–23). Under conditions of 200 mM KCl (\textit{cis})/50 mM KCl (\textit{trans}) with no divalent cations present in the extracellular solution (0 mM Mg), both H and L states are measured in stable single channel recordings, and they slowly and spontaneously interconvert. The M state is rarely observed (23). Under these conditions, the subconductance states of the CFTR channel represent a reproducible property of the channel pore. We compared the subconductance states of the \( \Delta R \)-CFTR chan-
channel with wild type to further test its pore properties. The 3-pS (L) subconductance state was also observed with the ΔR-CFTR channel. Fig. 7 shows consecutive single channel episodes obtained from a wild type (left panel) and a ΔR-CFTR channel (right panel), illustrating conversion of a single CFTR channel from the H to L conductance state. The L state of the ΔR-CFTR channel was similar to that of the wild type CFTR channel in terms of kinetics and current amplitudes, since both channels underwent fast transitions between the closed and partially open states once they occupied the L state. In a total of 74 successful experiments with the ΔR-CFTR channel, we observed 9 channels underwent transition from 8 to 3 pS, 6 channels underwent reverse transition from 3 to 8 pS, and 7 channels remained stable in the 3-pS conductance state without conversion to the 8-pS state within the time course of the bilayer experiments. The subconductance states of the ΔR-CFTR channel occurred at comparable frequency and converted with kinetics comparable with those of the wild type CFTR channel (23).

When the PP_i concentration was increased to above 15 mM, the open probability of the wild type CFTR channel increased, while that of the ΔR-CFTR channel did not change significantly.

Fig. 5. Effects of pyrophosphate on the wild type CFTR (WT-CFTR) and ΔR-CFTR channel. Single channel currents were taken from two separate experiments with the wild type CFTR and ΔR(708–835)-CFTR channels, following the addition of different concentrations of PP_i to the cis-solution (upper panels). The average open probabilities were plotted as a function of [PP_i] for the wild type CFTR (lower left panel) and ΔR(708–835)-CFTR (lower right panel). Each data point represents the average of 3–8 experiments. PP_i had a biphasic effect on the wild type CFTR channel, but it only had an inhibitory effect on the ΔR(708–835)-CFTR channel.

Fig. 6. Effects of vanadate on the wild type CFTR (WT-CFTR) and ΔR-CFTR channel. Representative single channel currents were obtained from a continuous experiment with the wild type CFTR channel (left panel) and the ΔR(708–835)-CFTR channel (right panel), following the addition of various concentrations of VO_4 to the cis-solution. VO_4 enhanced the activity of the wild type CFTR channel, but it inhibited the activity of the ΔR(708–835)-CFTR channel. Similar phenomena were observed in seven other experiments with the wild type CFTR channel and six other experiments with the ΔR(708–835)-CFTR channel.

WT-CFTR Control ΔR-CFTR

WT-CFTR

ΔR-CFTR

0 mM

0.5 mM

2 mM

15 mM

WT-CFTR Control ΔR-CFTR

WT-CFTR

ΔR-CFTR

0.4 mM VO_4

1 mM VO_4

4 mM VO_4

Fig. 4. Effect of AMP-PNP on the wild type CFTR and ΔR-CFTR channel. Representative single channel currents were taken from two separate experiments with the wild type CFTR (WT-CFTR, left panel) and ΔR(708–835)-CFTR channel (middle panel), before (Control) and after the addition of AMP-PNP (+2 mM AMP-PNP) to the cis-solution. The arithmetic mean open lifetime (t_{mean}) of the wild type (WT) CFTR channel increased from 96.0 ± 9.3 ms to 133.2 ± 15.6 ms following the addition of AMP-PNP (n = 4), while that of the ΔR(708–835)-CFTR channel did not change significantly. t_{mean} = 68.7 ± 5.2 ms (Control), 66.1 ± 7.8 ms (+AMP-PNP).

Fig. 5. Effect of pyrophosphate on the wild type CFTR (WT-CFTR) and ΔR-CFTR channel. Single channel currents were taken from two separate experiments with the wild type CFTR and ΔR(708–835)-CFTR channels, following the addition of different concentrations of PP_i to the cis-solution (upper panels). The average open probabilities were plotted as a function of [PP_i] for the wild type CFTR (lower left panel) and ΔR(708–835)-CFTR (lower right panel). Each data point represents the average of 3–8 experiments. PP_i had a biphasic effect on the wild type CFTR channel, but it only had an inhibitory effect on the ΔR(708–835)-CFTR channel.

Fig. 6. Effects of vanadate on the wild type CFTR (WT-CFTR) and ΔR-CFTR channel. Representative single channel currents were obtained from a continuous experiment with the wild type CFTR channel (left panel) and the ΔR(708–835)-CFTR channel (right panel), following the addition of various concentrations of VO_4 to the cis-solution. VO_4 enhanced the activity of the wild type CFTR channel, but it inhibited the activity of the ΔR(708–835)-CFTR channel. Similar phenomena were observed in seven other experiments with the wild type CFTR channel and six other experiments with the ΔR(708–835)-CFTR channel.
of the phosphorylated R domain protein did not change the
domain protein, within the same recording period. The addition
were obtained from the
D
lipid bilayer (unphosphorylated
domain protein are already present (Fig. 5), both the wild
wild type channel, did not affect the activity of the
exogenous R domain protein, from the same tube as can block the
product failed to affect the CFTR channel (12). However, exog-
consisting of reticulocyte lysate with irrelevant translation
P
protein results in an increase of the
VO4. However, the addition of phosphorylated R domain pro-
thesis and peptide-sequencing studies have identified five serines
(660, 700, 737, 795, and 813) as the major physiological targets
or the mutation of two or three in various combina-
was best fit with a
single exponential, for which the time constant was 58 ms for
open probability of the
D
R-CFTR channel in the presence of phosphorylated R domain and 2 mM PPi. Thus, there was no significant change in time constant with the addition of phosphorylated R domain or pyrophosphate. In particular, there was no evidence for bursting behavior.

DISCUSSION
Our data show that the R domain deletion mutant of CFTR,
ΔR(708–835), forms a functional chloride channel with normal
current properties and subconductance states, which does not require PKA phosphorylation to open. The
P
of the ΔR-
CFTR channel is about one-third that of the wild type CFTR
channel, whether or not it is phosphorylated. Unlike the wild
type channel, ΔR-CFTR cannot be blocked by exogenous un-
phosphorylated R domain or stimulated by AMP-PNP, PP
, or
VO4. However, the addition of phosphorylated R domain pro-
test results in an increase of the
P
of the ΔR-CFTR channel to
about two-thirds that of the wild type channel. Nevertheless, the burst duration remains brief.

The R domain of CFTR is highly hydrophilic, with ~28% of the
amino acids being charged residues. Site-directed mutagen-
esis and peptide-sequencing studies have identified five serines
(660, 700, 737, 795, and 813) as the major physiological targets
for phosphorylation by PKA, and the two best phosphorylation
sites (Ser737 and Ser813) are located within the region deleted in
ΔR(708–835) (27, 28). The multiple phosphorylation sites in
the R domain are redundant, since mutation of any one of them
to alanine or the mutation of two or three in various combina-
tions did not affect cAMP-dependent regulation of the CFTR
channel, while combined mutation of four of the serines, 660,
737, 795, and 813 (4SA), reduced the open probability of the
Fig. 8. Effect of the R domain on the ΔR-CFTR channel. A, sample traces from a ΔR-CFTR channel under control conditions, following the addition of RDP and following the addition of PKA to the cis-chamber containing the RDP. Records were filtered at 100 Hz. B, open probability of ΔR-CFTR channel. The first two bars show data from channels tested in the presence and absence of PKA. The second three bars show channels to which unphosphorylated R domain protein was added into the cis-chamber, with no change in open probability. However, when R domain protein was present, the addition of PKA increased the open probability approximately 2-fold.

Fig. 9. Effect of phosphorylated R domain protein on the open lifetime of the ΔR-CFTR channel. A, cumulative open time histograms were generated from 150 s of continuous recording at −80 mV from a single ΔR(708–835)-CFTR channel, before (Control) and after the addition of prephosphorylated R domain protein (+RDP(PKA)) to the cis-solution. The histograms were fitted with the following equation: $y = N(1 - \exp(-t/\tau_o))$, where $N_o = 253.6$, $\tau_o = 60.3$ ms (control); and $N_o = 425.3$, $\tau_o = 70.6$ ms (+RDP(PKA)). B, the arithmetic means of open times ($\tau_o$) were calculated from five paired experiments with the ΔR-CFTR channel before and after the addition of prephosphorylated R domain protein. $\tau_o = 48.9 \pm 16.2$ ms (control), 44.0 ± 15.4 ms (+RDP(PKA)). C, the number of open events was increased 2.32 ± 0.59-fold ($N_o/N_0$), following the addition of prephosphorylated R domain protein to the cis-solution.

CFTR channel by nearly 50% (10). More extensive combined mutations of up to nine serine residues and one threonine residue (10SA) led to further decrease in channel activity, but the channel still remained tightly controlled by PKA and ATP (8). The remaining regulatory site is serine 753, not a dibasic consensus PKA phosphorylation site (29).

By studying the function of the single ΔR-CFTR channel in the bilayer membrane, we confirm some of the previous studies of Rich et al. (9); ΔR-CFTR forms a chloride channel that does not require PKA phosphorylation to open. However, Rich et al. (9) reported that chloride efflux from cells transfected with ΔR-CFTR increased in response to cAMP stimulation, whereas we found no effect of PKA phosphorylation on the ΔR-CFTR activity. This apparent inconsistency probably results from the different systems used for study. The planar lipid bilayer assesses the effect of phosphorylation on single channel activity, whereas in whole cells, increased chloride efflux could result from increased delivery of functional channels to the cell surface as well. The complete lack of response of the ΔR-CFTR channel to PKA phosphorylation, despite the retention of the Ser660 site, which is sufficient to stimulate channel openings if the other major phosphorylation sites are mutated, suggests that the large deletion from the R domain prevents it from assuming a stimulatory conformation when it is phosphorylated. It is unlikely that the failure of $P_o$ to increase in response to PKA in the ΔR-CFTR channel is caused by active phosphatase activity, for in the same planar lipid bilayer system, when exogenous R domain protein is present, PKA causes $P_o$ to double. Moreover, when protein kinase inhibitor is added to phosphorylated wild type channels contained in HEK 293 microsomal vesicles, no rundown is observed over 10–20 min of observation, suggesting that phosphatase activity in HEK 293 vesicle preparations is minimal (12). If phosphatase is added to ΔR-CFTR channels, there is no decrease in $P_o$, indicating that this channel is not unresponsive to PKA because it is already fully phosphorylated by tonic PKA activity in HEK 293 cells or vesicles.

The role of the R domain in CFTR channel function may be clarified by these results. One possibility is that the R domain functions as a gating particle for the CFTR channel. This hypothesis is consistent with the observation that unphosphorylated R domain, added to the phosphorylated CFTR channel, prevents chloride transport and that elimination of the R domain in the ΔR-CFTR construct results in an open channel in vitro. However, it seems unlikely that the R domain operates like the gating particle described in some of the voltage-dependent ion channels (30, 31). Even the phosphorylated CFTR channel requires ATP hydrolysis to open (3), and exogenous
unphosphorylated R domain fails to block ΔR-CFTR, although its pore properties appear normal. On the other hand, the unphosphorylated R domain must prevent gating by ATP, because channels with an intact R domain (wild type) (5), or even an R domain containing up to nine serine to alanine mutations (8), do not open in the presence of ATP until activated by PKA. The nonhydrolyzable ATP analog 8-azido-ATP binds equally well to phosphorylated and unphosphorylated CFTR channels (32), suggesting that the unphosphorylated R domain does not interfere with ATP binding to the NBFs, although these studies (32) do not address the stoichiometry or sites of ATP binding. However, when a substantial portion of the R domain is deleted, either an inhibitory portion of it is also deleted or the conformation of R changes so that it can no longer inhibit. Although the mutant channel opens in the absence of PKA phosphorylation, channel kinetics are abnormal; \( P_o \) is low and openings are fewer and briefer compared with wild type channels.

One possible explanation for briefer openings is that the channel closes more quickly in the ΔR-CFTR mutant. One current hypothesis for the regulation of channel closure is that binding and hydrolysis of ATP at the second NBF effect channel closure, particularly from the bursting state. Several pieces of evidence suggest that NBF2 has an inhibitory role in the function of the CFTR channel. Sheppard et al. (33) showed that the N-terminal half of CFTR (Δ893X, a molecule without NBF2) forms an ATP-regulated chloride channel that opens in the absence of PKA phosphorylation and, once phosphorylated, exhibits higher \( P_o \) than the full-length CFTR channel (see Fig. 3 of Ref. 33). Point mutations within the conserved Walker A motif of NBF1 decreased the opening rate of the CFTR channel, while the corresponding mutations in NBF2 (K1250A, K1250M) prolong the open lifetime of CFTR (14, 15). The functional effects of K1250A and K1250M on the CFTR channel are similar to the effects of AMP-PNP and PP_i (19, 24), suggesting that a decrease in the ATP hydrolysis rate at NBF2 leads to prolonged opening of the CFTR channel. To test whether inhibiting activity at the NBF2 in the ΔR-CFTR mutant increased the \( P_o \), we applied several drugs that in wild type CFTR prolong burst duration, presumably by inhibiting ATP binding or hydrolysis at NBF2. However, neither AMP-PNP, PP_i, nor VO_4 increased \( P_o \) or burst duration in the ΔR-CFTR mutant. Thus, it is unlikely that the low \( P_o \) in this mutant is the result of increased inhibitory activity of NBF2. Alternatively, it may be that the ΔR-CFTR channel never achieves either high enough \( P_o \) or the bursting state from which it can be closed by binding and hydrolysis of ATP at NBF2, so changing the functional capacity of NBF2 has no effect on this channel. However, increasing \( P_o \) by the addition of phosphorylated R domain to 0.24 did not confer on \( P_o \) the ability to increase burst duration, so the lack of response to this agent in the ΔR-CFTR channel is probably not solely the result of low \( P_o \).

An alternative hypothesis is that in the ΔR-CFTR mutant, a normal stimulatory effect of the phosphorylated R domain has been eliminated. To test this possibility, we added phosphorylated exogenous R domain protein to the ΔR-CFTR channel in the planar lipid bilayer. This maneuver resulted in a nearly 2-fold increase in \( P_o \). Similar observations were made by Winter et al. (34). This increase resulted entirely from an increased number of channel openings, not increased burst duration. In addition, even when it was stimulated by exogenous phosphorylated R domain, the ΔR-CFTR channel did not respond to PP_i, with an increase in \( P_o \) or burst duration (time constant).

Our data and the data of others are consistent with a model for R domain function in which the unphosphorylated R domain exerts strong inhibition on either the ATP binding or hydrolysis or the transduction of their effect to open the channel pore. However, when the R domain is phosphorylated, it either stimulates ATP binding or hydrolysis or enhances transduction of their effect to open the channel. The inhibitory effect of R domain protein is relieved by phosphorylation, but if unphosphorylated R domain is available in excess, free in solution, inhibition can be effected. When a large portion of the R domain is deleted from CFTR, either the inhibitory portion is eliminated or the conformation of the remaining R domain is altered so that the inhibitory conformation cannot be assumed. The binding site for the unphosphorylated R domain is inaccessible to exogenous R domain in this configuration, possibly because the conformational change that occurs with phosphorylation is required to expose the inhibitory site to the solution or possibly because of constraints on the remaining amino acids in the shortened R domain channel. However, the ΔR-CFTR molecule, while lacking inhibition from the unphosphorylated R domain, also lacks the stimulatory effect of phosphorylated R...
domain. This effect can be reconstituted in part by exogenous phosphorylated R domain: this stimulatory site is accessible to occupation by exogenous protein in the ΔR-CFTR channel but not in the phosphorylated wild type channel, in which we speculate that endogenous phosphorylated R domain has the structural advantage. Exogenous phosphorylated R domain increases the opening rate but not open time. Bursting behavior may require a more exact conformational fit than exogenous R domain can supply. Even the addition of PP1 to the ΔR-CFTR channel in the presence of phosphorylated R domain does not increase open time.

The conformation of the R domain, as assessed by circular dichroism, changes with phosphorylation in solution in vitro (35). Such a change in conformation might allow the phosphorylated R domain to interact at a different site on NBF1 or in a different manner from the less charged, unphosphorylated version. The search for interaction among the protein domains of CFTR by physical means has been frustrating, but Price and co-workers (36) report interaction between NBF1 and the R domain. Our model predicts that interactions might also be detected between NBF1 and phosphorylated R domain.

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