Dibasic phosphorylation sites in the R domain of CFTR have stimulatory and inhibitory effects on channel activation

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Vais, Horia, Rugang Zhang, and William W. Reenstra. Dibasic phosphorylation sites in the R domain of CFTR have stimulatory and inhibitory effects on channel activation. Am J Physiol Cell Physiol 287: C737–C745, 2004. First published May 12, 2004; 10.1152/ajpcell.00504.2003.—To better understand the mechanisms by which PKA-dependent phosphorylation regulates CFTR channel activity, we have assayed open probabilities ($P_o$), mean open time, and mean closed time for a series of CFTR constructs with mutations at PKA phosphorylation sites in the regulatory (R) domain. Forskolin-stimulated channel activity was recorded in cell-attached and inside-out excised patches from transiently transfected Chinese hamster ovary cells. Wild-type CFTR and constructs with a single Ser-to-Ala mutation as well as octa (Ser-to-Ala mutations at 8 sites) and constructs with one or two Ala-to-Ser mutations were studied. In cell-attached patches, Ser-to-Ala mutations at amino acids 700, 795, and 813 decreased $P_o$, whereas Ser-to-Ala mutations at 737 and 768 increased $P_o$. In general, differences in $P_o$ were due to differences in mean closed time. For selected constructs with either high or low values of $P_o$, channel activity was measured in excised patches. With 1 mM ATP, $P_o$ was similar to that observed in cell-attached patches, but with 10 mM ATP, all constructs tested showed elevated $P_o$ values. ATP-dependent increases in $P_o$ were due to reductions in mean closed time. These results indicate that R-domain phosphorylation affects ATP binding and not the subsequent steps of hydrolysis and channel opening. A model was developed whereby R-domain phosphorylation, in a site-dependent manner, alters equilibrium between forms of CFTR with low and high affinities for ATP.

Phosphorylation during agonist-dependent activation. Results have differed between laboratories, but phosphorylation of serines at sites 660, 700, 737, 795, and 813 has been observed by several groups (11, 26, 27). While this does not demonstrate a role for these sites, these results decrease the likelihood that the other dibasic sites play a major role in the activation of wild-type CFTR. Studies in which serine has been mutated to alanine to prevent kinase-dependent phosphorylation have also been less than conclusive. No site appears to be essential for activation (11, 27). Removal of all dibasic sites reduces, but does not eliminate, PKA-dependent channel activity (8, 31). Studies in which single Ser-to-Ala mutations were made at dibasic sites have shown changes in the magnitude of channel activity, in the rate of channel activation, and in dose-response curves for agonist-dependent activation (4, 36, 37). The presence of serine at amino acid 737 or 768 appears to inhibit PKA-dependent channel activation. Serines at amino acid 660 or 813, and to a lesser extent 700 and 795, appear to stimulate channel activity. However, these studies had several flaws. Changes in apparent channel activity have not always been shown to be independent of CFTR expression. Changes in dose-response relationships do not permit a distinction to be made between changes in single-channel properties ($P_o$ or $i$) and changes in the amount of kinase activity needed to generate fully active channels. Last, how phosphorylation of CFTR solely at amino acid 737 or 768 affects channel activity is unknown. In general, there are three unresolved questions: (1)
At what site or combination of sites is phosphorylation required to produce a maximally active channel? 2) Are there sites in CFTR where phosphorylation inhibits channel activation? 3) How does CFTR phosphorylation permit channel activation?

To address these issues, we measured CFTR channel kinetics in cell-attached and excised patches for a series of constructs with Ser-to-Ala mutations at PKA phosphorylation sites in the R domain. In addition to examining the impact of single Ser-to-Ala mutations at PKA phosphorylation sites, we studied a mutant in which the eight conserved phosphorylation sites in the R domain were mutated to alanine and a series of constructs in which one or two of these alanines were mutated back to a serine. Our studies were designed to allow us to measure not only the open probability \( (P_o) \) but also mean open time \( (\tau_o) \) and mean closed time \( (\tau_c) \) for each of these mutants. Our studies demonstrate that phosphorylation at S737 or S768 inhibits channel activity by decreasing \( P_o \). Last, our studies demonstrate that phosphorylation alters the rate of channel opening and not the rate of channel closing. In contrast to the conclusions of a recent labeling study (5), our data suggest that R-domain phosphorylation alters an equilibrium between conformations of CFTR with high and low affinities for ATP. Similarities between this mechanism for CFTR activation and the mechanism for ATP-dependent solute absorption by bacterial ATP-binding cassette (ABC) transporters (10) are described.

**MATERIALS AND METHODS**

Generation of CFTR expression constructs with Ser-to-Ala mutations at phosphorylation sites in the R domain. We generated mammalian CFTR expression vectors in pcDNA3.1(+)(+) with single Ser-to-Ala mutations at PKA phosphorylation sites at 700 (700A), 737 (737A), 768 (768A), 795 (795A), and 813 (813A); with eight Ser-to-Ala mutations at 660, 686, 700, 712, 737, 768, 795, and 813 (octa); with seven Ser-to-Ala mutations at 700, 737, 768, and 795 (795S/813S); and with six Ser-to-Ala mutations and serines at 813 and 700 (700S/813S), 813 and 737 (737S/813S), 813 and 768 (768S/813S), and 813 and 795 (795S/813S) (Table 1). PTM-CFTR vectors were generated by site-directed mutagenesis by primer overlap was performed to introduce restriction sites downstream of S660 and S768, respectively. These PCR products were then cloned into pcDNA3.1-octa and pcDNA3.1-813S at the BsrGI and Hpal sites to generate all new vectors used in this study. All constructs were prepared with Endofree plasmid kits from Qiagen (Valencia, CA) and confirmed by restriction digestion and sequencing. All restriction enzymes were obtained from New England Biolabs (Beverly, MA).

**Cell culture and transformation.** Chinese hamster ovary (CHO-K1) cells were grown in Ham's F-12 nutrient mixture (Ham's medium; Cellgro-Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and 1% penicillin/streptomycin (10,000 U/ml; Cellgro-Mediatech). Cells were maintained in 150-mm plates, and the medium was changed every 2 days. Cells were passaged at 90–95% confluence and split 1 to 10. One day before infection, cells were plated onto glass coverslips (8–8 mm) in a 24-well plate. Approximately 2 \( \times 10^5 \) cells/well were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) and a 1:5 mixture of the green fluorescent protein-expressing vector pcDNA3.1-GFP and the wild-type vector pcDNA3.1-CFTR (or mutant forms described above). As suggested by the manufacturer, for 24 h, 0.3 \( \mu \)g of total DNA and 1 \( \mu \)l Lipofectamine were dissolved in 25 \( \mu \)l of serum-free Ham's F-12 medium, mixed, and incubated at room temperature for 45 min. Suspensions were then diluted with 150 \( \mu \)l of serum-free medium and spread onto rinsed cells. After 5 h, 400 \( \mu \)l of Ham's F-12 medium with 20% FBS was added to each well; after 24 h, cells were returned to standard medium.

**Electrophysiology.** Cells were used for patch clamping 1–3 days postinfection, with 2 days being optimal. Patch pipettes were pulled from PG52151-4 glass capillaries (World Precision Instruments, Sarasota, FL) using a vertical puller (PP-830; Narishige Instruments Laboratory, Tokyo, Japan). They had resistances of 4–6 \( \Omega \)M when filled with pipette solution of the following composition (in mM): 140 \( N \)-methyl-d-glucamine (NMDG)-Cl, 5 \( CaCl_2 \), 2 \( MgCl_2 \), and 10 HEPES, pH 7.4 (adjusted with NMDG). The bath solution was composed of (in mM) 145 \( NaCl \), 5 KCl, 2 \( CaCl_2 \), 2 \( MgCl_2 \), 5 glucose, and 5 HEPES, pH 7.4 (adjusted with NaOH). For inside-out patch experiments, the composition of the bath solution (IO buffer) was (in mM) 150 \( NaCl \), 2 \( MgCl_2 \), 5 EGTA, and 5 HEPES, pH 7.4 (adjusted with NaOH).

**Data acquisition.** Cell-attached and excised inside-out patch currents were recorded at room temperature (22°C) under constant membrane potentials, using an EPC-9 patch-clamp amplifier controlled with the associated PULSE software (Heka Electronic, Lambrecht, Germany). The apparent membrane potential (bath minus the pipette potential) was 50 mV. Data were sampled at 200 Hz, filtered at 100 Hz with a built-in three-pole Bessel filter, and saved directly to personal computer hard drive file for further offline analysis. Cells were patched in the absence of agonist, and, when patches with high-resistance seals (typically ~50 \( G_\Omega \)) and no basal channel activity were obtained, \( 1 \mu M \) forskolin was added to the bath. Channel activity was typically recorded for at least 20 min. For inside-out patch experiments, the patch pipette tip was repositioned after patch excision in front of a three-barrel pipette outlet from a fast-switching, gravity-driven (0.2 ml/min) perfusion system (SF77A; Warner Instrument, Hamden, CT) to allow the application of IO buffer supplemented with 50 U/ml PKA plus 1 \( mM \) ATP, 1 mM ATP, or 10 mM ATP. Excised patches were exposed to PKA plus ATP for 10 min before activity with 1 and 10 mM ATP was recorded. For most patches, the number of active channels \( (N) \), determined by variance analysis, remained constant in our experiments; patches for which a decrease in channel number was observed were not analyzed.

**Data analysis.** Data were digitally filtered at 10 Hz and analyzed using IGOR Pro software (WaveMetrics, Lake Oswego, OR).
records of at least 15-min duration for which the average current \( (I) \) was constant, \( P_o \) was determined using the equation \( P_o = \frac{(1 - \sigma^2 I - i)}{n} \), where \( \sigma^2 \) is the variance of \( I \) \((13)\). Single-channel current \( i \) was determined from amplitude histograms, which were also used to establish that baseline current was constant. Mean open times were calculated with the formula \( \tau_o = \frac{(T - i)}{(i - i_n)} \), where \( T \) is the length of the record \((\geq 180 \text{ s})\) and \( n \) is the total number of openings in the record \((24)\). Mean closed times were subsequently derived from \( P_o \) and \( \tau_o \). The number of channels in a patch \( (N) \) was calculated from \( \sigma^2, I, \text{and} \ i, N = \frac{((i - i_0)(1 - \sigma^2 I - i))}{i} \), and then compared with the maximum number of observed channels.

Reagents. Forskolin (Calbiochem, La Jolla, CA) was kept as stock solution \((10 \text{ mM})\) in DMSO at \(-20^\circ \text{C}\). Aliquots of PKA catalytic subunit (Promega, Madison, WI) were stored at \(-70^\circ \text{C}\) and suspended in IO buffer just before use. All other chemicals were obtained from Sigma (St. Louis, MO).

Statistics. Calculated values are given as means \pm SE. Data were compared using Student’s \( t \)-test, with \( P < 0.05 \) considered significant.

RESULTS

Expression constructs for CFTR containing mutations at PKA phosphorylation sites in the R domain were generated and expressed in CHO cells. The mutations studied are described in Table 1. In addition to wild-type CFTR and octa, in which eight PKA site serines were converted to alanine, we examined forms of CFTR with a single Ser-to-Ala mutation at a PKA phosphorylation site and forms of CFTR with Ser-to-Ala mutations at six or seven PKA phosphorylation sites. Cell-attached patches were established in the absence of agonist. After a stable baseline without interfering channel activity was established, 1 \( \mu \text{M} \) forskolin was added to the perfusion buffer and channel activity was recorded for up to 30 min; traces for wild type, octa, and 768A are shown in Fig. 1. Representative traces for wild type, octa, 813S, 768A, 768S, and 813S/768S are shown in Fig. 2A. Because the \( P_o \) values for CFTR and most of the mutants studied are \(<0.25\) and the mean open times are \( >1 \text{s} \), it is difficult to accurately determine the number of active channels in a patch \( (N) \) and, as a consequence, \( P_o \) values from the equation \( I = N \cdot i \cdot P_o \). We therefore chose to determine \( P_o \) from the single-channel current \( i \), the mean current \( I \), and the variance in mean current \( \sigma^2 \) with the following relationship: \( P_o = 1 - \sigma^2 i / I \) \((13)\). The use of this equation requires that \( i \) be measured accurately. To do this, we generated all-points current histograms and determined \( i \) from the average distance between adjacent peaks; a representative plot is shown in Fig. 3. A second requirement is that \( I \) be constant over the length of the record. This was established by calculating \( I \) for at least three intervals within a record and by determining that all values were no more than 10% larger than the smallest value of \( I \). When possible, baseline stability was assessed from the current levels of the all-closed state. When all-closed states were not observed, baseline stability was assessed by the absence of shoulders in the all-points histograms. For records for which these criteria could be established, \( P_o \) was calculated from \( \sigma^2 \). The results are shown in Table 2. Standard errors of the mean reflect multiple measurements of each record and are calculated from the uncertainty in the values of \( i, I, \text{and} \ \sigma^2 \). These uncertainties may not be insignificant, but because both \( i \) and \( \sigma^2 \) are proportional to \( i \), factors that alter \( i \) should not affect the calculated value of \( P_o \). Because \( P_o \) is calculated from a difference, there is a greater relative error in the low values of \( P_o \).

To convince ourselves that values of \( P_o \) calculated in this manner were real, we examined traces for 813S and octa, which are presented in Fig. 2A. For 813S and octa, \( I \) values were 0.284 and 0.288 \( \text{pA} \), respectively, but our analysis revealed values for \( P_o \) and \( N \) of 0.52 and 1.9, respectively, for 813S, while calculated values of \( P_o \) and \( N \) were 0.08 and 16, respectively. This difference is also seen in the all-points histograms in Fig. 3, in which there are clearly more active channels in the trace for octa and, consequently, \( P_o \) is smaller. It should be noted that for these traces, the values of \( i \), 813S (0.29 \( \text{pA} \)), and octa (0.22 \( \text{pA} \)) differed. This is not thought to be significant, because mean values of \( i \) for all 813S and octa patches studied were 0.28 \pm 0.05 and 0.23 \pm 0.03 \( \text{pA} \), respectively (Table 2). For all cell-attached patches studied, the mean value of \( i \) was 0.26 \pm 0.01 \( \text{pA} \) \((n = 67)\), and the distribution was normal.

During these studies, we made five recordings in which only a single active channel per patch appeared. For these records, \( P_o \) was calculated from all-points current histograms and from the variance in \( I \), and the ratio of \( P_o \) from variance analysis to \( P_o \) from all-points histograms was determined. The mean ratio was 0.95 \pm 0.06, suggesting that at least for single-channel patches, there were no significant differences between \( P_o \) calculated by variance analysis and from all-points histograms. We also compared the maximum number of channels observed in our traces with the number calculated from our \( P_o, i, \text{and} \ I \) values. While the maximum number of channels observed was often much less than the calculated number of channels, in none of the 67 patches analyzed was the maximum number of observed channels more than a fraction of a channel greater than the calculated number of channels. While these tests suggest to us that our values of \( P_o \) are correct, we acknowledge that factors unknown to us could have caused our analysis to generate spurious \( P_o \) values.

Our data show that single Ser-to-Ala mutations can have significant effects on \( P_o \), with 700A and 813A decreasing \( P_o \)
and 737A and 768A increasing $P_o$ relative to that of wild-type CFTR. However, in no case examined was phosphorylation at any one site essential for channel activity. This was also demonstrated by the observation that significant forskolin-dependent channel activity was observed with octa. The increased value of $P_o$ for 737A and 768A (relative to that of wild-type CFTR) suggested that phosphorylation at these sites might inhibit CFTR activation. However, an alternative explanation is that phosphorylation at these sites inhibits phosphorylation at activating sites. To distinguish between these explanations, single Ala-to-Ser mutations were generated in octa at

**Table 2. $P_o$ values for mutated CFTR**

<table>
<thead>
<tr>
<th>Construct</th>
<th>$P_o$ (pA)</th>
<th>$i$ (pA)</th>
<th>No. of Patches</th>
<th>Time, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.28±0.03p</td>
<td>0.23±0.02</td>
<td>5</td>
<td>1.110</td>
</tr>
<tr>
<td>700A</td>
<td>0.11±0.02</td>
<td>0.27±0.02</td>
<td>3</td>
<td>1.280</td>
</tr>
<tr>
<td>737A</td>
<td>0.39±0.03</td>
<td>0.29±0.02</td>
<td>5</td>
<td>1.590</td>
</tr>
<tr>
<td>768A</td>
<td>0.69±0.06</td>
<td>0.29±0.02</td>
<td>4</td>
<td>0.960</td>
</tr>
<tr>
<td>795A</td>
<td>0.16±0.07</td>
<td>0.26±0.05</td>
<td>3</td>
<td>1.240</td>
</tr>
<tr>
<td>813A</td>
<td>0.17±0.03</td>
<td>0.23±0.01</td>
<td>5</td>
<td>1.440</td>
</tr>
<tr>
<td>Octa</td>
<td>0.07±0.01</td>
<td>0.23±0.03</td>
<td>3</td>
<td>0.980</td>
</tr>
<tr>
<td>700S</td>
<td>0.27±0.04</td>
<td>0.30±0.02</td>
<td>5</td>
<td>1.780</td>
</tr>
<tr>
<td>737S</td>
<td>0.16±0.04</td>
<td>0.29±0.01</td>
<td>4</td>
<td>0.730</td>
</tr>
<tr>
<td>768S</td>
<td>0.08±0.02</td>
<td>0.24±0.04</td>
<td>4</td>
<td>1.270</td>
</tr>
<tr>
<td>795S</td>
<td>0.12±0.01</td>
<td>0.27±0.05</td>
<td>5</td>
<td>1.030</td>
</tr>
<tr>
<td>813S</td>
<td>0.42±0.04</td>
<td>0.26±0.05</td>
<td>4</td>
<td>1.990</td>
</tr>
<tr>
<td>700S/813S</td>
<td>0.45±0.03</td>
<td>0.24±0.05</td>
<td>3</td>
<td>0.500</td>
</tr>
<tr>
<td>737S/813S</td>
<td>0.25±0.03</td>
<td>0.26±0.02</td>
<td>5</td>
<td>0.920</td>
</tr>
<tr>
<td>768S/813S</td>
<td>0.15±0.02</td>
<td>0.29±0.01</td>
<td>4</td>
<td>1.220</td>
</tr>
<tr>
<td>795S/813S</td>
<td>0.62±0.03</td>
<td>0.24±0.01</td>
<td>5</td>
<td>1.090</td>
</tr>
</tbody>
</table>

$P_o$, open probability; $i$, single-channel current. *Total length of recordings analyzed; $p$ significant ($P < 0.05$) difference vs. octa; $s$ significant ($P < 0.05$) difference vs. wild type; $c$ significant ($P < 0.05$) difference vs. 813S; $d$ significant ($P < 0.05$) difference vs. corresponding single-mutation serine.
Phosphorylation of CFTR regulates ATP binding

Amino acids 700 (700S), 737 (737S), 768 (768S), 795 (795S), and 813 (813S). $P_o$ values for 737S and 768S were not significantly different from the $P_o$ value for octa. In contrast, the $P_o$ values for 700S, 795S, and 813S were significantly greater than the $P_o$ value for octa. Double-mutant constructs were generated from octa with one serine at 813 and a second serine at 700 (700S/813S), 737 (737S/813S), 768 (768S/813S), or 795 (795S/813S). For 768S/813S, the $P_o$ was significantly greater than that for 768S and significantly less than that for 813S. A similar trend was seen with 737S/813S, where the $P_o$ was significantly less than that of 813S; however, while the $P_o$ of the double mutant was greater than that of 737S, the difference failed to reach significance. In contrast, a double mutation at two stimulatory sites, 795S/813S, had a $P_o$ that was greater than that for either 795S or 813S when present alone.

As shown in the expanded traces in Fig. 2B, it was possible to identify each channel opening in these records. By counting channel openings per unit time and measuring both $i$ and $I$, mean open times were calculated. Mean closed times were then calculated from $P_o$ and the mean open time. Data are presented in Table 3. The method provides a measure of mean open time but provides no information about the distribution of mean open time. This is a limitation of the method, but it does not affect the validity of the calculated values. In addition, because mean open times are calculated from three readily obtainable parameters, they are likely to be more accurate than our $P_o$ values. For all but one form of CFTR tested (795S/813S), mean open times were not significantly different from those of wild-type CFTR. In 5 of 67 patches, we observed a single active channel. Because of the length of mean open and closed times, we were unable to obtain enough events for open- or closed-time histograms; however, for these patches, survivor times were generated from octa with one serine at 813 and a second serine at 700 (700S), 737 (737S), 768 (768S), 795 (795S), and 813 (813S). A similar trend was seen with 737S/813S, where the $P_o$ of the double mutant was greater than that of 737S, the difference failed to reach significance. In contrast, a double mutation at two stimulatory sites, 795S/813S, had a $P_o$ that was greater than that for either 795S or 813S when present alone.

Because mean open times were similar for all constructs studied, two possible mechanisms were considered for the longer closed times of octa and 768S: 1) the rate of ATP binding could be reduced, or 2) the rate of channel opening with bound ATP could be reduced. If the longer closed times were due to differences in the rate of ATP binding and not the rate of channel opening once ATP was bound, mean closed times at high ATP concentrations should have been the same for all of our constructs. If the variation in closed times were due to differences in the rates of channel opening once ATP was bound, closed times would vary at all concentrations of ATP. To test this hypothesis, channel kinetics of wild type, octa, 768S, and 813S were studied in excised patches. Cells were patched and stimulated with forskolin as described for cell-attached patches. After channel activation, ATP and PKA were added to the bath and patches were excised. Representative traces from these experiments are shown in Fig. 5A. Traces at left show patches bathed in 1 mM ATP; traces at right are for the same patches in the presence of 10 mM ATP. $P_o$ data at both 1 and 10 mM ATP are shown in Fig. 5B. As in cell-attached patches (Table 2), at 1 mM ATP the $P_o$ values of octa and 768S were significantly less that those of wild type and 813S. However, at 10 mM ATP, there was no significant difference between the $P_o$ values of any construct. In Table 4, data for mean open and closed times are shown. Mean open times were not significantly different for any of the constructs and were affected little by increasing ATP concentration. The observed changes in $P_o$ for octa and 768S reflect changes in mean closed times. The data suggest that at elevated ATP concentrations, where most channels have bound nucleotides, the rates of channel opening and closing are unaffected by differences in R-domain phosphorylation.

**DISCUSSION**

The primary purpose of this study was to test the hypothesis that phosphorylation at S737 and S768 inhibits PKA-dependent stimulation. This hypothesis, based on dose-response relationships for IBMX-dependent increases in forskolin-stimulated CFTR activity, was originally suggested by Wilkinson et al. (36). Relative to wild-type CFTR, 737A and 768A had decreased $k_{1/2}$ for IBMX-dependent stimulation. Because it is unlikely under the conditions used (10 mM forskolin) that IBMX increased cAMP concentration or PKA activity (2), and binding with bound ATP could be reduced. If the longer closed times were due to differences in the rate of ATP binding and not the rate of channel opening once ATP was bound, mean closed times at high ATP concentrations should have been the same for all of our constructs. If the variation in closed times were due to differences in the rates of channel opening once ATP was bound, closed times would vary at all concentrations of ATP. To test this hypothesis, channel kinetics of wild type, octa, 768S, and 813S were studied in excised patches. Cells were patched and stimulated with forskolin as described for cell-attached patches. After channel activation, ATP and PKA were added to the bath and patches were excised. Representative traces from these experiments are shown in Fig. 5A. Traces at left show patches bathed in 1 mM ATP; traces at right are for the same patches in the presence of 10 mM ATP. $P_o$ data at both 1 and 10 mM ATP are shown in Fig. 5B. As in cell-attached patches (Table 2), at 1 mM ATP the $P_o$ values of octa and 768S were significantly less that those of wild type and 813S. However, at 10 mM ATP, there was no significant difference between the $P_o$ values of any construct. In Table 4, data for mean open and closed times are shown. Mean open times were not significantly different for any of the constructs and were affected little by increasing ATP concentration. The observed changes in $P_o$ for octa and 768S reflect changes in mean closed times. The data suggest that at elevated ATP concentrations, where most channels have bound nucleotides, the rates of channel opening and closing are unaffected by differences in R-domain phosphorylation.

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because IBMX has been shown to stimulate CFTR channel activity by a direct interaction (2, 16), it is not clear that the effects of IBMX were on PKA-dependent activation. Channel phosphorylation was not measured, and it was assumed that mutations at one phosphorylation site did not alter agonist-dependent phosphorylation at other sites. However, if phosphorylation at one site were required before phosphorylation at activating sites could occur, then mutations at S813 and S700 could inhibit channel activity, not because phosphorylation at these sites is stimulatory but because the absence of phosphorylation at these sites blocks phosphorylation at activating sites. Conversely, phosphorylation at 737S or 768S might inhibit CFTR activity by blocking phosphorylation at activating sites. An interplay of phosphorylation sites is made more likely by the presence of eight conserved PKA sites in the R domain.

Studies by Baldursson et al. (4) showed that in Fisher rat thyroid epithelial cells, cAMP-dependent chloride currents are greater for cells transfected with 737A than for cells transfected with wild-type CFTR. However, those authors failed to determine whether 737A expression was greater than that of wild-type CFTR, and only a small, apparently statistically insignificant difference in the k_{1/2} of dose-response curves for wild type and 737A was observed. With excised patches, Winter and Welsh (37) compared the P_0 values for wild-type CFTR with those for 737A, an inhibitory site mutation, and 660A, 795A, and 813A, activating site mutations. At low ATP concentrations, the P_0 of wild-type CFTR was greater than the P_0 for all other forms, including 737A; but at high ATP concentrations, there were no significant differences in P_0 for all forms of CFTR. On the basis of these studies, we concluded that the evidence for inhibitory effects of phosphorylation at S737 and S768 was not convincing.

Can better evidence for inhibitory effects of phosphorylation at S737 and S768 be obtained? The phosphorylation state of

Table 4. Mean open and closed times in excised patches

<table>
<thead>
<tr>
<th>Construct</th>
<th>ATP, 1 mM</th>
<th>ATP, 10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_0</td>
<td>(\tau_c) s</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.41±0.04</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>813S</td>
<td>0.46±0.03</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Octa</td>
<td>0.14±0.03*</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>768S</td>
<td>0.05±0.01*</td>
<td>0.8±0.2</td>
</tr>
</tbody>
</table>

*Significant (P < 0.05) difference vs. wild type.

Fig. 5. Current recordings for excised patches of CFTR and site-directed CFTR mutant channels expressed in CHO cells. A: cell-attached patches were formed and channels were activated with 1.0 \(\mu\)M forskolin. Cells were then perfused with IO buffer containing 1 mM ATP. Patches were excised and exposed to 1 mM ATP and PKA (50 U/ml) for 10 min before channel activity was assayed in the presence of 1 mM ATP (left) and 10 mM ATP (right). Representative traces for wild-type, octa, 813S, and 768S are shown. B: graphed data are means ± SE for P_0 in the presence of 1 mM ATP (light bars) and 10 mM ATP (dark bars). * P_0 values significantly different from those for wild-type CFTR.

C742 PHOSPHORYLATION OF CFTR REGULATES ATP BINDING

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channels in a patch can never be determined, because in vivo labeling does not allow this issue to be resolved. As a consequence, the only measurable property is agonist-dependent CFTR activity. While differences between constructs that differ from wild-type CFTR by one Ser-to-Ala mutation are clearly due, at least in part, to differences in phosphorylation at the mutated site, additional effects due to alterations in phosphorylation at other sites cannot be ruled out. We attempted to circumvent this problem by limiting the number of PKA sites. We studied both Ser-to-Ala mutations on a wild-type background and Ala-to-Ser mutations on a background in which the eight conserved PKA sites in the R domain of CFTR were mutated to alanine, octa. As described by others (27, 31), a small level of forskolin-dependent channel activity was seen with octa. Moreover, the activity of 737S and 768S was significantly greater than that of octa, while that of 700S, 795S, and 813S was significantly greater than that of octa. When there is only a single phosphorylation site, forskolin-dependent activity cannot be due only to phosphorylation at that site. For constructs with two phosphorylation sites, channel activity of the double mutant differs from that of both single-mutant constructs, both PKA sites must be phosphorylated in the double-mutant construct. A construct with an activating site and an inhibiting site should have an intermediate level of activity between the levels of the two single-site constructs. The PM of 813S/768S was less than that for 813S but greater than that for 768S, and similar effects were seen with 813S/737S. It is clear that, at least in the double mutations, phosphorylation occurs at S737 and S768 and that phosphorylation at these sites inhibits CFTR activity. Similarly, a construct with two activating sites should have channel activity that is greater than that of either single-site construct. Because the PM of 813S/795S is greater than that for either 813S or 795S, phosphorylation at both of these sites must be stimulatory. On the basis of this argument, we conclude that in our CFTR constructs, forskolin-dependent changes in channel activity were due to phosphorylation at the mutated sites. Our study also documents the presence of sites on CFTR where PKA-dependent phosphorylation is inhibitory. The presence of stimulatory and inhibitory phosphorylation sites may provide a mechanism for more precise regulation of CFTR channel activity.

While there is general agreement that R-domain phosphorylation is required before ATP can open CFTR channels, there is far less agreement with respect to how this is achieved. Two mechanisms have been proposed: 1) an R-domain blocking mechanism whereby an unphosphorylated R domain blocks ATP binding or hydrolysis and, conversely, a phosphorylated R domain could promote ATP binding (23, 37); and 2) a coupling mechanism whereby R-domain phosphorylation is required to couple ATP binding and hydrolysis to channel opening and closing (5, 20). The R-domain blocking mechanism is supported by observations made in several laboratories that deletion of the R domain produces a channel (R less CFTR) that has ATP-dependent but PKA-independent gating activity (23, 28). Moreover, the addition of PKA-treated R domain increased the channel activity of R-less-CFTR (23, 25, 37). The R-domain coupling mechanism, while difficult to reconcile with gating by R-less-CFTR, is supported by the observation that ATP analogs can photolabel CFTR in the absence of R-domain phosphorylation (5, 34). In addition, R-domain phosphorylation alters the Km for ATP hydrolysis (20). Because cellular ATP concentrations are well in excess of the reported Km values, these data suggest that phosphorylation would have minimal effects on the rate of ATP hydrolysis under in vivo conditions. While these results can be viewed as supporting the second mechanism, they are not inconsistent with the first mechanism. If R-domain phosphorylation were to alter the affinity of CFTR for ATP, it would increase the Vmax/Km for ATP hydrolysis without changing Vmax. Phosphorylation would also increase the rate, but not the extent, of photolabeling. Because the rate of photolabelling has not been reported (5, 34), these studies do not rule out the blocking mechanism.

While there is considerable controversy with regard to the mechanisms that control CFTR gating, most models incorporate the following features: 1) kinase-dependent phosphorylation, most likely on the R domain; 2) channel gating coupled to ATP binding or hydrolysis; and 3) channel closing upon the loss of nucleotide. A kinetic schema incorporating these features is shown in Fig. 6. While the model cannot describe features of the channel that require multiple ATP binding sites (1, 5), it is sufficient for the purposes of this discussion. As
shown in Fig. 6A, two conformations of the unliganded CFTR are envisioned: one, a closed form CFTRc, that can bind ATP and one, an inactive form CFTRi, that cannot bind ATP. Phosphorylation shifts the equilibrium between CFTRc and CFTRi with phosphorylation at activating sites favoring CFTRc and phosphorylation at inhibiting sites favoring CFTRi. Dephosphorylation of activating and inhibiting sites by phosphatases has the opposite effect. The effects of individual phosphorylations are additive, so that phosphorylation on multiple activating sites shifts CFTRc/CFTRi equilibrium further toward CFTRc than does phosphorylation at a single activating site. However, at sufficiently high concentrations of ATP, all CFTR has bound ATP, regardless of the level of phosphorylation. Channel gating (Fig. 6B) is envisioned as involving four states: two closed states, one with bound ATP and one without bound ATP; and two open states, one with bound ATP and one with bound ADP-Pi. The open channel with bound ATP is necessitated by recent studies demonstrating channel gating in the presence of nonhydrolyzable ATP analogs, albeit with \( P_{\text{c}} < 5\% \) of that for ATP (1, 35). While only one hydrolysis cycle is shown, a similar cycle exists for each of the closed forms of CFTR in Fig. 6A. The individual rate constants for each of these cycles may or may not differ from one another. In the presence of forskolin, or PKA for excised patches, our CFTR mutants are assumed to represent partially phosphorylated forms of CFTR, with intermediate values for the CFTRc/CFTRi equilibrium constant between those for unphosphorylated and fully phosphorylated CFTR. Both the R-domain blocking and R-domain coupling mechanisms are compatible with this model. If CFTRi is unable to bind ATP, the model describes the R-domain blocking mechanism; but if ATP can be bound and hydrolyzed by CFTRi without generating an open channel, this model describes the R-domain coupling mechanism.

The model in Fig. 6 allows several predictions to be made. The apparent rate constant for ATP binding to CFTR, \( k_{\text{on}} \), will be a function of the concentration of CFTRc (CFTRc+CFTRi)/CFTRc, and therefore of the level of phosphorylation. As a consequence, mutated forms of CFTR that act as models for partially phosphorylated forms of CFTR can have altered apparent rates for ATP binding to CFTR that are due to changes in CFTRc concentration and not to changes in \( k_{1} \). Provided that \( k_{3} = 0 \) for activation by nonhydrolyzable analogs of ATP, the fact that the \( P_{\text{o}} \) with ATP is greater than the \( P_{\text{o}} \) with nonhydrolyzable analogs while mean open times are not significantly different requires that \( k_{-2} \gg k_{2}, k_{3} \gg (k_{4} + k_{-3}) \), and \( k_{4} \approx k_{-2} \). As a consequence for ATP, the open channel is largely in the ADP-Pi form and the mean open time is equal to \( 1/k_{4} \). Because the mean open times for our mutants were not significantly different from those of wild-type CFTR, the rate of product dissociation, \( k_{4} \), does not appear to be altered by channel phosphorylation. The mean closed time is equal to \( ([ATP] \cdot k_{1} + (k_{-1} + k_{-2})/\text{[ATP]} \cdot k_{1} \cdot k_{2} \). Because mean closed time varies with CFTR phosphorylation, either the rate of ATP binding, \( k_{1} \), or the rate of channel opening in the presence of bound ATP, \( k_{2} \), must vary with channel phosphorylation. Winter and Welsh (37) demonstrated that, at high ATP concentrations for which mean open time is equal to \( 1/k_{2} \), the mean closed times for wild-type CFTR, 660A, 737A, 795A, and 813A were not significantly different. We have shown that at high ATP concentrations, the mean closed times of wild type, octa, 813S, and 768S were not significantly different. The observations at high ATP concentrations are most consistent with phosphorylation not affecting \( k_{2} \), the rate of channel opening with bound ATP. Changes in mean closed times at low ATP concentrations and under cell-attached conditions are most consistent with phosphorylation altering the fraction of CFTR in the CFTRc form and not altering \( k_{1} \). Given these observations, models such as the one shown in Fig. 6 require that changes in mean closed time for our constructs were due to differences in the concentration of CFTRc and not in \( k_{1} \), the rate constant for ATP binding to this form of the channel.

CFTR is a member of the ABC transporter superfamily. All other members of this family are thought to be ATP-dependent pumps that translocate substrates across, from, or within cell membranes. The mechanism for CFTR activity proposed in Fig. 6 is analogous to that for ABC transporters. One well-studied example is the maltose transporter in gram-negative bacteria (10, 12). A periplasmic binding protein, MalE, undergoes a conformational change upon binding maltose with high affinity. In the bound conformation, MalE binds to extracellular regions of a closed maltose transporter (MalFGK2). This induces a conformational change in the NBDs (MalK), allowing ATP to be bound and/or hydrolyzed. ATP hydrolysis causes a second conformational change in the transmembrane domains (MalFG) that 1) opens a transmembrane passageway for substrate entry into the cell and 2) distorts the conformation of MalE, thereby reducing its affinity for the substrate. Substrate is then able to enter the cell, and the release of nucleotide returns the transporter to the closed conformation, which is unable to bind unliganded MalE. Similar mechanisms are likely used by ABC transporters that bind substrate directly. For CFTR, phosphorylation of the R domain is analogous to the binding of liganded MalE. This allows ATP binding and/or hydrolysis to alter the conformation of the TMDs and open a channel. The channel remains open until nucleotide is released. For the maltose transporter, substrate transport is coupled to ATP hydrolysis by the fact that only when liganded MalE is present can ATP hydrolysis open the passageway, and the presence of the binding protein prevents the loss of ligand during transport. For CFTR, the R domain does not block the channel, and chloride can move into or out of the cell while the channel is open. Also, because the release of nucleotides does not dephosphorylate the R domain, multiple channel openings, each of which is coupled to ATP hydrolysis, are possible without rephosphorylating the R domain.

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