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Identification and Regulation of the Cystic Fibrosis Transmembrane Conductance Regulator–Generated Chloride Channel

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Abstract

Cystic fibrosis transmembrane conductance regulator (CFTR) generates cAMP-regulated Cl− channels; mutations in CFTR cause defective Cl− channel function in cystic fibrosis epithelia. We used the patch-clamp technique to determine the single channel properties of Cl− channels in cells expressing recombinant CFTR. In cell-attached patches, an increase in cellular cAMP reversibly activated the low conductance Cl− channels. cAMP-dependent regulation is due to phosphorylation, because the catalytic subunit of cAMP-dependent protein kinase plus ATP reversibly activated the channel in excised, cell-free patches of membrane. In symmetrical Cl− solutions, the channel had a channel conductance of 10.4±0.2 (σ = 7) pS and a linear current–voltage relation. The channel was more permeable to Cl− than to I− and showed no appreciable time-dependent voltage effects. These biophysical properties are consistent with macroscopic studies of Cl− channels in single cells expressing CFTR and in the apical membrane of secretory epithelia. Identification of the single channel characteristics of CFTR-generated channels allows further studies of their regulation and the mechanism of ion permeation. (J. Clin. Invest. 1991. 88:1422–1431.) Key words: cystic fibrosis • patch-clamp • phosphorylation • Cl− secretion • cAMP

Introduction

Cystic fibrosis (CF)† is a genetic disease (1), characterized by defective function of Cl− channels in affected epithelia: in CF secretory epithelia, an increase in intracellular cAMP fails to open apical membrane Cl− channels (1, 2). Expression of wild-type CF transmembrane conductance regulator (CFTR) (3) in CF epithelia corrects the defect in cAMP-regulated Cl− permeability (4, 5). Moreover, expression of CFTR generates cAMP-regulated Cl− channels in a variety of cells that do not normally express CFTR and do not normally have cAMP-regulated Cl− channels (6, 7). These observations are explained by recent studies which demonstrate that CFTR is itself a cAMP-regulated Cl− channel: mutation of amino acids in the membrane spanning sequences of CFTR alter its anion selectivity (8).

Previous studies showing that CFTR generates Cl− channels relied primarily on the whole cell patch-clamp technique to measure macroscopic channel currents (6–8). However, it is also important to define the single channel properties, so that they can be compared to those of previously reported channels, and so that the excised, patch-clamp technique can be used in future studies of channel biophysics and regulation. To examine the single channel properties, we expressed CFTR in cells that do not normally express CFTR and do not normally have cAMP-regulated Cl− channels (6). Two different cell types (HeLa cells and 3T3 fibroblasts) and two different expression systems (transient and stable) were used.

We also asked whether CFTR-generated Cl− channels are regulated by phosphorylation with the cAMP-dependent protein kinase (PKA). Several observations suggested that PKA-dependent phosphorylation would regulate this Cl− channel: the amino acid sequence of CFTR has multiple potential phosphorylation sites for PKA (3); CFTR is a phosphoprotein (9); an increase in intracellular cAMP activates Cl− channels studied with the whole cell patch-clamp technique (4–8); and deletion of the majority of the potential phosphorylation sites generates channels that are open even without an increase in cAMP (9). Those observations did not, however, tell us whether phosphorylation activated the Cl− channel or whether cAMP itself might regulate the Cl− channel in a manner analogous to cyclic nucleotide-gated channels in photoreceptors and olfactory receptors (10). To test the hypothesis that the CFTR-generated Cl− channels are regulated by phosphorylation, we examined the effect of the catalytic subunit of cAMP-dependent protein kinase on Cl− channels in excised, cell-free patches.

Methods

Cells and transfection procedure. We used HeLa cells and NIH 3T3 fibroblasts; in previous studies we have shown that these cells do not express CFTR (by polymerase chain reaction, by immunoprecipitation, or by function [6, 11]). To transiently express CFTR in HeLa

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1. Abbreviations used in this paper: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; IBMX, 3-isobutyl-1-methyl-xanthine; NMDG-Cl, N-methyl-D-glucamine chloride; PKA, protein kinase; TES, N-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

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Figure 1. Expression of CFTR induces cAMP-activated Cl⁻ currents in cell-attached patches from 3T3-CFTR fibroblasts. (A) Time course of changes in current produced by addition of 100 μM IBMX and 20 μM forskolin (cAMP, as indicated by bars). Membrane voltage was maintained at -40 mV and stepped to +40 mV for 1 s every 6 s. Data points are average current obtained during the 1-s pulse to +40 mV. *Time at which I-V relation in C was obtained. (B) Current traces obtained at the times indicated by the lower case letters in A. All traces were obtained at +40 mV, two traces are shown for each condition. Scale bar for traces in b are 30 pA and 3 pA for all other traces. Dashed line indicates zero current. (C) I-V relation from cell-attached patches. I-V relation indicated by closed symbols is from data shown in Fig. 1, A and B. Pipette solution contained 142 mM Cl⁻ and 140 mM NMDG. I-V relation indicated by open symbols was obtained from a different cell in which pipette contained 15.5 mM Cl⁻. For both traces, basal currents (before addition of cAMP) were subtracted from values after addition of forskolin and IBMX.
cells, we used the vaccinia virus-T7 hybrid expression system developed by Elroy-Stein and co-workers (12). We have previously described the methods and the evidence that this system produces functional CFTR (4, 6, 11). Briefly, HeLa cells were plated at ~5 x 10⁴ cells/cm² on collagen-coated plastic coverslips 24 h before infection. Recombinant vaccinia virus vTF7-3 (10–20 multiplicity of infection) was added to the cells for 1 h in serum-free media, and then cells were transfected with recombinant plasmids (5 μg of plasmid/10⁶ cells) with lipofectin (20 μg of lipid/10⁶ cells) and incubated at 37°C. Cells were studied 10–18 h after transfection.

We also studied NIH 3T3 fibroblasts stably expressing CFTR (3T3-CFTR cells). CFTR cDNA (4.5 kb from the initiating ATG to SalI site at nucleotide position 4622) was subcloned into a retroviral vector, the properties of which will be published elsewhere (Thompson, S., and R. C. Mulligan, submitted for publication). The resultant plasmid was cotransfected with pSV2 neo (13) into ψCRE cells (14). 48 h after transfection, cells were selected with G418 (1 mg/ml). Medium harvested from G418-resistant cells was used to infect NIH 3T3 fibroblasts with a single 4-h exposure. The efficiency of infection was determined by Southern analysis: ~ 80% of the NIH 3T3 cell population was found to contain an intact proviral genome. As a control, NIH 3T3 fibroblasts were exposed to medium harvested from nontransfected ψCRE cells.

**Patch-clamp technique.** The methods used for single channel patch-clamp recording are similar to those previously described (15). Cells and bath were maintained at 30–35°C by a temperature-controlled microscope stage (Brook Industries, Lake Villa, IL). Pipette resistance was 2.5–6 MΩ and seal resistance was 2–25 GΩ. In some cases higher resistance pipettes (4.4–12.7 MΩ) and seals (3–110 GΩ) were used in an attempt to decrease the number of channels per patch. An amplifier (EPC 7; List Biological Laboratories, Campbell, CA) was used for current amplification and voltage clamping, and a laboratory computer system (Indec Systems Inc., Sunnyvale, CA) was used for data acquisition.

**Figure 2.** Expression of CFTR induces cAMP-regulated Cl⁻ currents in cell-attached patches HeLa cells. (A) Time course of activation in response to 100 μM IBMX and 20 μM forskolin (present during times indicated by bar cAMP). Pipette contained 14 mM Cl⁻, 136 mM aspartic acid, and 140 mM NMDG. Data points indicate average current during a 1-s pulse to −60 mV from a holding voltage of 0 mV. (B) Representative traces obtained at the times indicated by the lower case letters in A. Voltage was −60 mV. Dashed line indicates zero current. (C) I-V relation obtained at the time indicated by the asterisk in A. The estimated reversal potential for a Cl⁻ channel was +68 mV. Basal current was subtracted.
acquisition and analysis. Current amplitudes were determined from amplitude histograms. In current–voltage (I-V) relations, lines were fit either by eye or by linear regression. Currents were filtered at 1 kHz and data was digitized at 2 kHz. Voltages are reported in reference to the external surface of the membrane and outward (+) current refers to the flow of anions from the external to internal surface of the patch.

Chemicals and solutions. 3-isobutyl-1-methyl-xanthine (IBMX), forskolin, and adenosine 5'-triphosphate magnesium salt were obtained from Sigma Chemical Co., St. Louis, MO. Two types of the catalytic subunit of cAMP-dependent protein kinase were used with similar results. Catalytic subunit of PKA was obtained from Promega Biotec, Madison, WI. Recombinant catalytic subunit alpha of PKA was prepared using a full-length cDNA encoding the Chinese hamster enzyme (16) which was inserted into a bacterial expression vector (17). Recombinat catalytic subunit purified from the bacterial expression system was homogeneous as determined by polyacrylamide gel electrophoresis and exhibited a $K_{m}$ of 18.6 μM for Kemptide with a $V_{max}$ of 10 μmol/min per mg.

Pipette solutions contained (in mM): NaCl or N-methyl-d-glucamine chloride (NMDG-Cl) (140), MgCl$_2$ (1.2), CaCl$_2$ (1.2), N-Tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) (10), pH = 7.2. In a few experiments the concentration of MgCl$_2$ was 5 mM and CaCl$_2$ was 2 mM. Bath solutions contained (in mM): NaCl, NaI, or NMDG-Cl (135), MgCl$_2$ (2.4), EGTA (1), TES (10), dextrose (10), indomethacin (3 μM), pH = 7.2. Aspartic acid replaced Cl$^{-}$ in bath and pipette solutions containing low Cl$^{-}$. Final Cl$^{-}$ concentrations are indicated in the text or figure legends for the individual experiments.

Results and Discussion

Studies of cell-attached patches. A defining characteristic of macroscopic Cl$^{-}$ currents generated by expression of CFTR is their regulation by cAMP (4–8). Thus, to determine the single channel basis of the macroscopic Cl$^{-}$ current, we first asked whether an increase in intracellular cAMP would activate Cl$^{-}$ channels in cell-attached membrane patches.

When we added 20 μM forskolin and 100 μM IBMX (to increase intracellular levels of cAMP), Cl$^{-}$ currents increased in 3T3-CFTR fibroblasts. Fig. 1 A shows a representative time course: current began to activate within 40 s of adding forskolin and IBMX and rapidly plateaued. When the agonists were removed, current returned to basal values within 100 s. Current could be reversibly activated on repeated addition of agonist (Fig. 1 A). In 17 of 18 patches from 3T3-CFTR cells, cAMP increased current by 39±27 pA (mean±SEM) within 77±52 s. When agonists were removed, current returned to baseline in 10 of 10 patches. A second stimulation with cAMP increased current in two of two experiments.

The tracings in Fig. 1 B show that cAMP-activated currents appeared noisy and often wavy, suggesting that multiple, low conductance channels were present in the membrane patch. Similar results were obtained with CFTR expressed in insect cells (7). In such tracings it was not possible to discern discrete single channel events; however, examination of the reversal potential in I-V relations, indicated that current flowed through Cl$^{-}$ channels. Fig. 1 C shows the I-V relation when the pipette contained 142 mM Cl$^{-}$; the reversal potential was 3±3 mV (in five experiments with these solutions). If the current were carried by Cl$^{-}$, we would expect a reversal potential near this value, based on an estimated intracellular Cl$^{-}$ concentration of 40 mM and an estimated membrane voltage of -40 mV (18). Rectification in the I-V relation at negative voltages is consistent with a lower intracellular than extracellular Cl$^{-}$ concentration. When the pipette solution Cl$^{-}$ concentration was

![Figure 3](image-url)

Figure 3. Single-channel currents from a cell-attached patch in a 3T3-CFTR fibroblast. Pipette solution contained 140 mM NMDG-Cl (estimated reversal potential for a Cl$^{-}$ channel was 0 mV). (A) Cell-attached, single channel tracings at the voltages indicated. (B) I-V relation of channel in A. Single-channel conductance was 8.6 pS at positive voltages and 6.2 pS at negative voltages.
Figure 4. Activation of Cl⁻ channels in an excised, inside-out patch from a 3T3-CFTR fibroblast. External (pipette) solution contained 142 mM Cl⁻ and 140 mM NMDG; bath contained 140 mM Cl⁻ and 141 mM Na⁺. (A) Time course for activation and reversal of currents; bars indicate times during which catalytic subunit of PKA (75 nM) and ATP (1 mM) were present in the internal solution. *Time during which data shown in Fig. 8 was obtained. Data are average currents during a 1-s pulse to +40 mV from a holding voltage of 0 mV. (B) Representative current traces obtained at the times indicated by lowercase letters in A.
reduced from 142 to 15.5 mM, the reversal potential shifted to 
+47±1 mV (n = 5) as expected for a Cl⁻ selective channel (Fig. 1 C). In contrast, when Na⁺ was substituted for NMDG in the pipette solution, the reversal potential did not change (n = 6, not shown).

We found similar results in HeLa cells transiently expressing CFTR. Fig. 2 A shows that forskolin and IBMX reversibly stimulated current. Fig. 2 B shows representative current tracings; as observed with 3T3-CFTR fibroblasts, multiple channels were contained in the patch and single channels were not readily resolved. IBMX and forskolin activated current within 67±10 s in 3 of 12 cell-attached patches and the effect was reversible in 2 of the 3 patches. This frequency of expression is consistent with the heterogeneous vaccinia virus/T7 expression system (4, 6). Fig. 2 C shows that the I-V relation reversed at a positive voltage, as expected for a Cl⁻ selective channel (the pipette contained 14 mM Cl⁻; the estimated reversal potential for a Cl⁻ channel was +68 mV).

Although most patches contained multiple channels, we did observe single channel currents in six cell-attached patches from 3T3-CFTR cells stimulated with forskolin and IBMX (no channel activity was observed before stimulation). Fig. 3 A shows representative current tracings; Fig. 3 B shows the single channel I-V relation. In cell-attached patches with a pipette Cl⁻ concentration of 140 mM, the single channel conductance at positive voltages was 8.6±0.3 pS and at negative voltages was 5.6±1.4 pS (n = 5).

These data indicate that cAMP reversibly activated a low conductance Cl⁻ channel in cell-attached membrane patches.

Studies of excised, cell-free patches. To more precisely determine the conductive properties of CFTR-generated Cl⁻ channels and to investigate their regulation by phosphorylation, we turned to excised, cell-free patches. Fig. 4 A shows the time course of current activation in a patch from a 3T3-CFTR cell. Addition of ATP (1 mM) did not activate current during 130 s of observation, but within 34 s of adding the catalytic subunit of PKA (in the continued presence of ATP), current increased. Fig. 4 B shows representative current tracings obtained at the times indicated in Fig. 4 A. The currents had a wavy, noisy pattern consistent with the presence of multiple channels in the patch and similar to what we had observed in cell-attached patches. Activation required the combination of the catalytic subunit of PKA and ATP; addition of either alone was insufficient. When we removed PKA and ATP, current decreased to basal levels. During basal conditions, we only rarely observed channel openings; Fig. 4 B (parts c and e) show examples. When we once again added PKA and ATP to the cytosolic surface of the patch, the channels reactivated. PKA and ATP activated channels in 40 of 55 patches with current increasing by 25±21 pA. Activation reversed when the agonists were removed in 27 of 27 patches and a second stimulation increased current in 13 of 13 patches. The average time from addition of PKA and ATP until channels were activated was 73±71 s.

It is interesting that the second addition of PKA and ATP activated less Cl⁻ current than the first. We observed the same phenomena on increasing intracellular cAMP in cell-attached patches (Fig. 1 A) and in whole cell patch-clamp studies (6).

Fig. 5 shows I-V relations from a multichannel excised, inside-out patch. In the presence of symmetrical and asymmetrical Cl⁻ concentrations, the reversal potential shifted as expected for a Cl⁻-selective channel.

Just as with cell-attached patches, it was difficult to obtain excised patches that contained a small enough number of channels to observe single channel events. To reduce the number of channels per patch, we used smaller pipettes and were then able to observe single channel events in some cases. Fig. 6 A shows an example from a patch in which at least three channels were activated within 85 s after addition of ATP and catalytic subunit of PKA.

The I-V relation for the single channel (Fig. 6 B) was linear in symmetrical Cl⁻ concentrations and the single channel conductance was 10 pS. Using high resistance pipettes, we observed single channel events at a complete series of voltages in seven patches activated by PKA and ATP; average single channel conductance was 10.4±0.2 pS². Fig. 6 B also shows that when the intracellular (bath) solution was exchanged for a low Cl⁻ concentration (n = 4), the reversal potential shifted as expected for a Cl⁻-selective channel. Because of the small single channel conductance, we could not reliably determine the reversal potential from single channel traces; reversal potentials and selectivity are more reliably determined from the multichannel patches. We were also sometimes able to observe single channel events in patches containing a large number of chan-

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2. Single channel conductance (7.4±0.5 pS, n = 5) was lower in a 10-mM Hepes buffer than in a TES buffer. The inhibitory effect of Hepes on Cl⁻ channels has been reported previously (27, 28).
A

100 mV
80 mV
60 mV
40 mV
20 mV
0 mV
-20 mV
-40 mV
-60 mV
-80 mV
-100 mV
1 pA
102.4 ms

Figure 6. Single channel current activated by PKA and ATP in an excised, inside-out patch. (A) Tracings were obtained from a 3T3-CFTR fibroblast after addition of catalytic subunit of PKA (75 nM) and ATP (1 mM); no channel activity was observed before addition of both agents. Tracings were obtained at the indicated voltages. Pipette contained 142 mM Cl− and 140 mM NMDG; bath contained 139 mM Cl− and 144 mM NMDG. (B) I-V relation for channel shown in A is shown by solid symbols. Open symbols show data obtained after reduction of bath Cl− concentration to 14 mM (Cl− was replaced by aspartic acid, expected reversal potential for Cl− current is −62 mV).

channels: after we removed forskolin and IBMX from cell-attached patches or PKA and ATP from excised patches, we sometimes saw unitary events as the current decreased toward basal levels. These currents always had the same conductive and permeability properties as those observed in patches that only contained a few channels.3

3. Given the values of single-channel conductance, the minimum number of channels required to produce the increase in current with stimulation was 85±62 channels in cell-attached patches and 63±52 channels in excised patches. The actual number of channels was obviously much higher because the open state probability was <1. Because of the substantial number of channels per patch we could not accurately determine open state probability or number of channels.

Fig. 7 A shows that the channel was selective for Cl−: substitution of internal Na+ for NMDG did not alter the I-V relation. We found similar results in multichannel patches (n = 8). We previously showed that the CFTR-generated macroscopic Cl− currents in the whole cell were more permeable to Cl− than to I− (8); Fig. 7 A and B, shows that this was also the case for channels activated by PKA in excised patches. The calculated selectivity ratio of I− to Cl− (P/I/PCl−) was 0.42±0.06 (n = 3); in good agreement with values obtained from whole cell and apical membrane studies: P/I/PCl− was ~ 0.57 (8). In addition, as we previously reported for whole-cell studies (8), the conductance was less in I− than in Cl−.

Channels activated in excised patches by PKA and ATP showed no discernible time-dependent voltage effects. Fig. 8
shows individual traces from a multichannel patch (Fig. 8 A) and an ensemble average (Fig. 8 B) of such traces taken during steps to ±100 mV from a holding voltage of 0 mV. There is no time-dependent increase or decrease in current at either voltage. Similar results were obtained in three patches and also when voltage-dependence was tested at ±80 mV or at ±60 mV. Similar results were obtained with cell-attached patches (not shown). These results are consistent with studies of CFTR-generated currents using the whole cell patch-clamp technique: minimal, if any, voltage-dependence was observed (4–8). We did not observe activation of Cl⁻ channels by membrane depolarization, as has been observed with outwardly rectifying Cl⁻ channels (19).

Our previous studies using the whole cell patch-clamp technique and a fluorescent microscopic technique showed that 3T3 fibroblasts and HEla cells do not normally contain cAMP-regulated Cl⁻ channels (6). That observation was confirmed in cell-attached patches (an increase in cAMP failed to elicit Cl⁻ current in 3 of 3 patches from control 3T3 fibroblasts, compared to 1 of 17 experiments on 3T3-CFTR cells, \( P = 0.003 \) by chi-square analysis), and in excised patches (PKA and ATP failed to elicit Cl⁻ currents in 4 of 4 patches, compared with 15 of 55 patches from 3T3-CFTR cells, \( P < 0.02 \).

Relationship of CFTR-generated Cl⁻ channels to other Cl⁻ channels. Some of the single-channel properties of CFTR which we report are similar to those reported by Kartner et al. (7); when CFTR was expressed in insect cells, they found that the I-V relation was linear with a single channel conductance of 8.4 pS, the channel was observed more frequently in cAMP-stimulated cells, and \( \Gamma^- \) was more permeable than Cl⁻ (\( P_I/P_Cl^- \)...

Figure 7. I-V relations of Cl⁻ channels stimulated with PKA and ATP in excised patches. (A) Data are from single channel currents. Pipette contained 142 mM Cl⁻/140 mM NMDG; bath (internal solution) contained either 140 mM Cl⁻/141 mM Na (solid symbols) or 135 mM I/143 mM Na (open symbols). (B) Data are from a multichannel patch. Pipette contained 142 mM Cl⁻/140 mM NMDG; bath contained 135 mM I/143 mM Na. \( P_I/P_Cl^- \) was 0.34.
Moreover, mutation of CFTR altered its anion selectivity (8). The single channel properties of CFTR-generated Cl− channel, which we report here, are different from those of the outwardly rectifying Cl− channel (reviewed in [19]). The CFTR-generated Cl− channel has a linear I-V relation and single channel conductance of 10 pS, whereas the outwardly rectifying Cl− channel has a single channel conductance of ~30–50 pS. In addition, the outwardly rectifying Cl− channel is more permeable to I− than to Cl−, whereas the CFTR Cl− channel is more permeable to Cl− than to I− (8, 20). The outwardly rectifying Cl− channel is also activated by prolonged periods of strong membrane depolarization: after activation by strong membrane depolarization the channel remains in an activated state. In contrast, CFTR-generated Cl− channels were not activated by membrane depolarization; they were only activated by cAMP in cell-attached patches or by the catalytic subunit of PKA in excised patches. Moreover, such activation was reversible. Thus, the CFTR-generated Cl− channel and the outwardly rectifying Cl− channel are most likely two different channels. How then can one reconcile previous studies showing defective regulation of the outwardly rectifying Cl− channel in CF epithelia with the CFTR Cl− channel? We do not know the answer. One possibility is that physiologic abnormalities resulting from mutations in CFTR somehow produce defective (secondary) regulation of other channels. A problem that is perhaps similar to understanding how mutations in CFTR (i.e., a defect in Cl− channel function) produce the wide variety of other phenotypic manifestations in CF.

The CFTR-generated Cl− channel also has different properties from Ca2+-activated and volume-activated Cl− channels in epithelia (20, 21): the most obvious differences are in regulation and anion selectivity. Of note, the CFTR-generated Cl− channels are not dependent on internal Ca2+ (our internal solution was Ca2+-free), an observation consistent with previous observations that Ca2+ and cAMP activate two different types of apical membrane Cl− channels (20).

The properties of CFTR-generated Cl− channels are similar to those of an 8.6-pS channel with a linear I-V relation studied in cell-attached patches from T84 cells (23). That channel was observed with a higher frequency in patches from cAMP-stimulated cells and it had no time-dependent voltage effects, but anion selectivity was not determined. A 4–7-pS channel with a linear I-V was also observed in pancreatic duct epithelium (24–25). That channel was stimulated by cAMP in cell-attached patches, but the anion selectivity was different: Br− ≈ I− ≈ Cl−. Other Cl− channels have also been reported (26), but their relationship to the present data is uncertain.

Summary. These data identify the single channel properties of CFTR-generated Cl− channels. The channel has a conductance of ~10 pS, displays a linear I-V relation when studied in symmetrical Cl− concentrations, shows minimal time-dependent voltage effects, and is more permeable to Cl− than to I−. Our data also indicate that these channels are activated by phosphorylation with PKA. These data set the stage for further studies into the biophysical properties of CFTR and its regulation.

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