Cystic fibrosis transmembrane conductance regulator (CFTR), which causes cystic fibrosis when nonfunctional, is an anion channel and a member of the ATP binding cassette superfamily. After phosphorylation, CFTR gates by binding and hydrolyzing ATP. We show that CFTR open probability ($P_o$) also depends on the electrolyte concentration of the cytosol. Inside-out patches from Calu-3 cells were transiently exposed to solutions of 160 mM salt or solutions in which up to 90% of the salt was replaced by nonionic osmolytes such as sucrose. In lowered salt solutions, CFTR $P_o$ declined within 1 s to a stable lower value that depended on the electrolyte concentration, ($K_{1/2}$ ≈ 80 mM NaCl). $P_o$ was rapidly restored in normal salt concentrations without regard to the electrolyte species. Reducing external electrolytes did not affect CFTR $P_o$. The same results were obtained when CFTR was stably phosphorylated with adenosine 5′-O-(thiotriphosphate). The decrease in $P_o$ resulted entirely from an increase in mean closed time. Increasing ATP levels up to 20-fold did not counteract the effect of low electrolytes. The same effect was observed for CFTR expressed in C127 cells but not for a different species of anion channel. Cytosolic electrolytes are an unsuspected, essential cofactor for CFTR gating.

We have discovered an unsuspected and powerful influence on CFTR gating. Our data indicate that CFTR senses the concentrations of cytosolic electrolytes (apparently without regard to species) and requires high levels of electrolytes (∼160 mM) for full activity.

**MATERIALS AND METHODS**

**Cells and Cell Culture**—Experiments were conducted using Calu-3 cells grown as previously described by Shen et al. (18) and CFTR-transfected C127 cells (2WT2 cells) provided by Seng Cheng (Genzyme Corp.) and grown as described by Haws et al. (19).

**Patch Clamp Recording and Solutions**—Inside-out single-channel patch clamp recordings were made at ∼23°C. CFTR channels were identified by their signature: unitary conductance of 6.1 ± 0.2 pS ($n$ = 8, mean ± S.E.), linear current-voltage relations, appeared in clusters, showed no voltage sensitivity, and required PKA and ATP for activity. They were the only anion channels recorded in cell-attached patches from surrounded cells in confluent islands of Calu-3 cells and were by far the most prevalent channels in transfected C127 cells (2WT2). Excised patches were alternately exposed to different flowing cytosolic solutions via solution changers (see below). CFTR activity was maintained with a perfusate containing 160 mM NaCl, 2 mM Mg$^{2+}$-ATP, and 10 units of PKA and was eliminated with a perfusate that lacked ATP and PKA. Other solutions contained the same activation ingredients and various concentrations of electrolytes or nonelectrolytes. Pipettes were pulled from Prism LA16/SA16 glass (Dagan Corp.) on a Brown-Flaming puller. Currents were amplified and filtered at 100 Hz with an Axopatch 1C amplifier. Data acquisition was controlled by pClamp 8.0 (Axon Instruments). Data were digitized at 1 kHz and stored on disk.

Membrane voltage was held at 60 mV unless otherwise indicated.

The standard bath and cytosolic control solution was (in mM) 150 NaCl, 5 KCl, 0.5 EGTA, 2.5 MgCl$_2$, 0.227 CaCl$_2$, and 10 HEPES titrated to pH 7.3 with ∼6.2 mM NaOH. Osmolarity was adjusted to 330–350 mosmol. The free calcium level was 100 nM as computed by MaxChela-tor. This formulation gives an electrolyte concentration of ∼34 mM; the main anion is Cl$^-$ (160 mM). Mg$^{2+}$-ATP or Na$_2$ATP (0.5–10 mM) was added to the stock, and the pH was adjusted to 7.3 by adding ∼1 mM NaOH/mM ATP. To avoid changes in $P_o$ caused by dephosphorylation via patch-associated phosphatas, all solutions in most experiments contained both Mg$^{2+}$-ATP and 10 units of the catalytic subunit of PKA with 50 mg/ml dithiothreitol. Test solutions were identical, except that NaCl and KCl were replaced with NaCl of the desired concentration plus the osmotically balanced replacement solute. The standard pipette solution was (in mM) 150 NMHD-Cl, 2.5 CaCl$_2$, 2.5 MgCl$_2$, and 10 HEPES, with pH adjusted to 7.3 with NaOH and osmolarity adjusted to 320–340 mosmol.

Because most test solutions contained reduced levels of Cl$^-$, recordings were usually made at positive cell voltages to provide the clearest channel recordings. Poor channel resolution was seen at negative voltages because of the unfavorable gradient for chloride and a fast block by anions (20). With reduced cytosolic Cl$^-$, the channel amplitude was increased less at positive voltages than the Nernstian prediction. This effect presumably resulted from channel saturation and block by the osmolytes (20).

**Chemicals**—Reagents were from Sigma unless otherwise noted. Forskolin was stored as a 10 mM stock solution in ethanol at −20°C. PKA stock was made from 1000 units of solid dissolved in 1 ml of deionized water containing 50 mg/ml dithiothreitol and stored at −20°C. ATP (Mg$^{2+}$ and Na$^+$ salt) and ATPγS (BioChemika) were dissolved in deionized water and stored at −10°C. Test solutions were made by replacing part of the NaCl with 2 parts of the nonelectrolytes sucrose, mannitol...
(Mallinkrodt), L-proline and or taurine or 1 part of the electrolytes Na-gluconate and Na-isethionate. In experiments in which Na$_2$SO$_4$ replaced NaCl, the electrolyte concentrations were balanced.

Solution-changing Apparatus—A rapid solution-switching system was fabricated using a piezoelectric translator (21) connected to an array of three parallel perfusion tubes, each ~100 μm in diameter and containing a different solution. The excised patch was positioned in relation to the array such that any of three computer-controlled voltages moved the array to bathe the patch in one of the streams. Switching between streams occurred in ~50 ms. Patches were alternately exposed to an activating solution containing PKA, 1–5 mM Mg$^{2+}$, ATP, and normal bath electrolytes; to a minimal solution that lacked PKA and ATP; and to test solutions that were equivalent except that varying proportions of cytosolic NaCl were replaced with nonelectrolytes or other electrolytes.

The valve-controlled solution changer consisted of a manifold with seven inlet ports, each connected to a different solution reservoir, and one outlet port connected to a perfusion pipette. The manifold was located ~2 mm from the tip of the perfusion pipette. Solutions were switched manually in ~2 s. In this setup the patch and perfusion pipette have fixed positions, which assures identical access to each solution.

Data Analysis—All-point amplitude histograms were constructed for selected traces to determine the amplitude of the unitary current. Histograms were least squares fit with ($N + 1$) Gaussian functions ($N$, number of active channels in the patch; resolution $N < 10$). The resulting average peak-to-peak interval represented the unitary current ($i_o$). $P_o$ was determined through least squares fits of binomial distributions of the multiple Gaussians or by $P_o = I/i_{NT}$, where $I$ is the integrated current, and $T$ is the total recording time. Mean closed time ($t_c$) was estimated by multiplying $N$ times the mean closed time of full closures (the record contained at least 10 full closures). Mean open time ($t_o$) was derived from the equation $P_o = t_o/ (t_o + t_c)$. $N$ was also determined by the peak current observed in a patch, divided by the unitary current obtained as above.

RESULTS

When cytosolic NaCl was rapidly switched from 160 to 15 mM with replacement by an iso-osmolar amount of sucrose, the $P_o$ of CFTR declined to ~20% of control values within 1 s. $P_o$ returned to normal with a similar time course after switching back to the control cytosolic solution (Fig. 1a). An equivalent reduction of $P_o$ was observed regardless of the species of nonelectrolyte that we used for replacement, including the cyclic sugar sucrase, the linear sugar mannitol, or the amino acids L-proline and taurine (Fig. 1b). (Proline is uncharged at neutral pH, and only ~3% of taurine is predicted to be charged at pH 7.4.)

This surprising result could be explained by many variables, including increased molar concentration of a nonelectrolyte, decreased molar concentration of Na$^+$, Cl$^-$, Na$^+$ or Cl$^-$, or electrolytes, or decreased ionic strength. Adding 320 mM mannitol to 160 mM NaCl did not reduce $P_o$ (Figs. 1c and 2a), indicating that the nonelectrolytes were not themselves inhibitory. (The current amplitude in the presence of 300 mM mannitol was reduced from 0.47 to 0.43 pA. This result is consistent with that of Linsdell and Hanrahan (20), who found that internal 220 mM sucrose or urea caused a 5–10% reduction in unitary conductance.)

CFTR $P_o$ was also unaffected by solutions in which Na$^+$, Cl$^-$, or both were replaced with different ions, including the impermeant anions gluconate, sulfate, and isethionate and the impermeant cation N-methyl-$\alpha$-glucamine (Figs. 1d and 2b). Taken together, these experiments appear to eliminate all variables except molar concentrations of electrolytes or ionic strength. Experiments with divalent ions will be needed to discriminate between those possibilities. In the remainder of the paper we use the term “electrolyte concentration” as an inclusive term for either molar concentrations of electrolytes or ionic strength.

To determine the quantitative dependence of CFTR $P_o$ on electrolyte concentration, the cytosolic solution bathing a single excised patch was rapidly switched between control solution with 160 mM NaCl and different test solutions containing 5, 15, 30, 60, 80, or 320 mM NaCl. For most of these experiments, the nonelectrolyte was proline. Open probability in these experiments was a sigmoid function of log salt concentration, with the steepest slope occurring between 60 and 100 mM and a half-maximal value of ~80 mM NaCl (Fig. 3).

In experiments to this point, the extracellular (pipette) solution was 150 mM NMDG-Cl. To determine whether the concentration of external electrolytes or the electrolyte gradient across the patch influenced CFTR $P_o$, we measured $P_o$ in a series of experiments in which ~1 mm of the pipette tip was filled with 150 mM NMDG-Cl and then back-filled with 15 mM NaCl + 300 mM proline, while the bath remained constant at 160 mM NaCl. In prior experiments with the nonpermeant anion ATP, we found that the tip solution equilibrates with the back-fill solution over a few minutes (22). In our present experiments, when tips were filled with 150 NMDG-Cl and back-filled with 15 Cl, the unitary current at 60 mV decreased by 25% within 1 min and remained stable. Under these conditions CFTR $P_o$ remained constant ($P_o = 0.35–0.42$; < 3.8% reduction; $n = 3$) for >15 min. We also studied two patches in which the extracellular solution was 60 mM NaCl and the cytosolic solution was switched among 160, 80, 30, and 15 mM NaCl. We saw the same decline as when the external solution was 160 mM.
NMDG-Cl. These experiments indicate that $P_o$ depends on the electrolyte concentration of the cytosol and not on external electrolyte concentration or the gradient of electrolytes across the membrane.

To determine the kinetic basis of the electrolyte dependence of CFTR activity ($NP_o$), we first needed to determine whether channel number ($N$) was altered in lower electrolyte solutions. Because of the smooth functions relating $NP_o$ to electrolyte concentration and the rapid onset and reversal of the changes in activity, we suspected that $N$ did not change, and that was confirmed when $N$ was estimated for each patch as described under "Materials and Methods." To assess $N$ more directly, we searched our records for instances of multiple channel openings during periods when the reduction of electrolytes caused a reduction in $NP_o$. As shown by the example in Fig. 4, by this criterion we concluded that $N$ was essentially unchanged in the two conditions. We also considered the alternate possibility that reduction in $NP_o$ was caused entirely by a change in $N$. For that to be true, the ratio of apparent $P_o$ in solutions of high and low electrolyte concentrations should be directly related to the ratio of $N$ in high- and low-salt concentrations. As shown in Fig. 4C, the ratio of $N$ was almost invariant and was close to 1 for $>10$-fold change in $P_o$.

Based on this evidence that $N$ was constant across electrolyte concentrations, we proceeded to estimate channel open and closed times in solutions of different electrolyte concentrations.
CFTR Gating Requires Cytosolic Electrolytes

Using the strategy outlined under "Materials and Methods." This analysis, (shown for a single patch containing four channels in Fig. 5, a and b), indicated that the low P_o observed in 15 mM NaCl solutions resulted from a >10-fold increase in mean closed time with no detectable effect on mean open time. For this patch, control versus 15 mM NaCl values were P_o, 0.30 versus 0.021; open time (s), 0.591 versus 0.512; and closed time, 1.52 versus 23.87.

The same results were obtained after analysis of four additional patches exposed to 160 and 30 mM NaCl. For those four patches, the mean open time in low-electrolyte solution was 0.99 ± 0.20 of the control value, whereas the closed time was 3.95 ± 0.9 times longer than the control value. We conclude that electrolyte levels affect channel closed time and not N or open time.

In addition to this multichannel analysis, we searched for single-channel patches. We eventually obtained one example of a "pseudo" single-channel patch, in which two CFTR channels were active in the cell-attached configuration, but after excision one channel became nearly silent, so that double openings constituted only 0.0005 of the total open time. We exposed the patch to control solution and to 60 mM NaCl balanced osmotically with taurine and analyzed the dominantly active channel as a single channel. Probability density distributions (Fig. 5, c and d) were computed for open and closed time in the control (531 events) and 60 mM NaCl (368 events) solutions. The distributions were best fit with two exponentials with the time constants shown in the figures. This analysis confirmed that open time was unchanged and also revealed that only the slow component of the closed time was changed in 60 mM NaCl, increasing from 0.6 to 1.4 s.

CFTR activity is affected by the cytosolic pH, which alters phosphatases and kinases and hence the phosphorylation and dephosphorylation of CFTR (12). To determine whether electrolytes might also affect the phosphorylation state of CFTR, we studied CFTR after stable phosphorylation with ATP-γ-S and found that the P_o of CFTR was still decreased and then restored to normal when switching between control and low-electrolyte concentrations in the absence of PKA. For these experiments, P_o values in 160 and 15 NaCl were 0.31 ± 0.05 and 0.05 ± 0.02, respectively (n = 5; p < 0.01; Fig. 6). The P_o and mean open and mean closed times were derived for one patch phosphorylated with ATP-γ-S and indicate that the effect was again entirely due to a change in the mean closed time. For 160 mM NaCl the values were: P_o, 0.19; t_o, 0.22 s; and t_c, 0.91 s. For 15 mM NaCl the values were P_o, 0.01; t_o, 0.21 s; and t_c, 27.63 s. (Note that this 30-fold change in closed time occurred in a patch estimated to have only four channels.) These results suggest that the electrolyte effect is most likely mediated via postphosphorylation gating events.

The constellation of effects observed so far distinguishes the electrolyte effect from most other factors known to affect CFTR gating but is similar (although scaled by a concentration factor of ~1000) to the dependence of P_o on ATP concentration, which also decreases the mean closed time with no effect on mean open time (23). To determine whether the reduced opening rate of CFTR in low cytosolic electrolytes resulted from reduced binding of ATP, we varied the ATP level 20-fold, from 0.5 to 10 mM, and measured P_o in 15 mM NaCl. Na2ATP was used, with Mg2+ held constant at 2.5 mM and pH adjusted to 7.3 with NaOH. (This process caused Mg-ATP levels to vary only 5-fold, from 0.5 to 2.5.) Higher levels of Mg2+ were not used because of known effects on CFTR gating (13). Higher ATP levels did not

![Phosphorylated with ATP-γ-S](image_url)

FIG. 6. The electrolyte effect was not mediated via phosphorylation or dephosphorylation of CFTR. After stable phosphorylation with ATP-γ-S, PKA was removed, and the patch was alternately exposed to solutions containing 2 mM ATP and either 160 or 15 mM NaCl (proline substitution). Top trace, continuous recording showing one cycle of solution changes. Bottom traces, expanded portions of channel activity in each condition. Bars indicate the regions that were expanded.
The discovery that CFTR gating depends on the electrolyte concentration of the cytosol was completely unexpected. In two prior studies using reduced electrolytes with sucrose substitution, no effects on $P_o$ were described (20, 27). In those studies the electrolyte level was decreased to 75 mM, a value that in our studies reduced $P_o$ only to approximately half of control values. Because of inherent variations in CFTR $P_o$ (13, 28), it is unlikely that a decline of that magnitude would be noticed unless a rapid solution switching system were used. Although no effect on $P_o$ was noted, it is interesting that a reduction in CFTR channel conductance by intracellular sucrose was observed (20, 27). We also observed a reduction in channel conductance when using uncharged intracellular osmolytes (see “Materials and Methods”), which tended to offset the expected increase in channel current predicted by the greater driving force for Cl$^-$. We found no other reports in which channel gating was shown to require cytosolic electrolytes. Many prior studies show influences of electrolytes on gating, but these are all distinct from the present phenomenon. For example, volume-regulated channels are affected by ionic strength (29), but the sign is opposite from what we observe. The gating of many channels is influenced by specific ions. Examples include a strong affect of extracellular K$^+$ concentration on the voltage dependence of an inwardly rectifying cardiac K$^+$ current (30) and the gating of an outward-rectifying K$^+$ channel in plant stomatal guard cells (31). Gating ($NP$) of the rat epithelial Na$^+$ channel was significantly decreased when intracellular [Na$^+$] was increased from 0 to 50 mM in inside-out patches (32), and CLC channel gating is influenced in a complex manner by the concentration and species of internal and external anions (33). These phenomena are distinct in their features from the present findings. They all depend on specific ions, two are voltage-dependent, and the epithelial Na$^+$ channel effect has a sign opposite to what we observed. Also, in these examples ion levels modulate gating, whereas CFTR gating has an absolute dependence on electrolytes. We also found only rare examples in which the transport or ATPase activity of ABC transporters was shown to be affected by electrolyte concentration. For example, ATPase activity and histidine transport of histidine permease are salt-sensitive, but the pattern differed markedly from what we observed, being high at 0 NaCl, maximal at ~50 mM, and then declining at higher concentrations (34, 35).

Is electrolyte concentration a physiological regulator of CFTR? It is conceivable that the steep dependence of CFTR $P_o$ on the electrolyte concentration of the cytosol could regulate CFTR-mediated conductance under some physiological conditions, such as in the sweat duct, where it has been hypothesized that a salt sensor closes CFTR at low luminal NaCl values (12, 36). The electrolyte dependence of CFTR $P_o$ could allow it to be the salt sensor if the extremely high conductance of the duct apical membrane (~125 mS/cm$^2$; Ref. 37) coupled the NaCl concentrations of cytosol and lumen. However, the sweat duct is exceptional in having a very high apical membrane conductance, a very low concentration (~15 mM) of luminal ions, and low water permeability. In most cells and conditions, the cytosolic electrolyte concentration should be sufficient to maintain high CFTR activity, and alterations in external electrolyte concentration would probably affect only cell volume. These considerations suggest that electrolytes are, like Mg$^{2+}$ and ATP, essential and typically invariant cofactors for CFTR activity.

How might electrolytes alter CFTR gating? Kinetic analysis distinguishes the electrolyte effect from other factors known to affect CFTR gating, such as the species and concentrations of divalent cations (10, 13, 14) and the redox status (14). Our data also suggest that an altered phosphorylation state does not explain the electrolyte effect, although more work needs to be done on that point. Low electrolytes did not affect channel open time. Because of considerable evidence that the open time is determined by hydrolysis at NBD2 (6, 38), the unchanged open time indicates that hydrolysis of ATP at NBD2 and all subsequent steps leading to closing are not directly affected by electrolyte levels. The remaining possibilities are that the electrolyte concentration affects either ATP binding at the NBD, ATP

**Fig. 7.** The electrolyte effect was observed for CFTR expressed in other cell types but not for another type of ion channel. a, the $P_o$ of CFTR exogenously expressed in C127 mouse mammary cells was dependent on the cytosolic electrolyte level. b, two outwardly rectifying, depolarization-induced chloride channels exposed to reduced electrolytes with sucrose replacement. c, equivalent results for a single outwardly rectifying, depolarization-induced chloride channel with mannitol replacement. In each patch unitary currents were partially blocked by the nonelectrolyte, but $P_o$ was unaffected. Dashed lines indicate closed state (note offset).
hydrolysis at NBD1, or the linkage between these events and channel opening. Our preliminary results do not support an electrolyte effect on ATP binding, although those experiments do not definitively rule out such an effect, because when we varied the ATP levels, we also varied the electrolyte concentration. Although the possibility of an electrolyte effect on ATP binding is still open, it appears to be more likely that electrolytes are affecting some aspect of ATP hydrolysis at NBD1 or the consequences following from hydrolysis.

How might electrolytes have such effects? Two general kinds of explanations seem salient. First, electrolytes may be required for the structural integrity of CFTR. An effect on CFTR structure might arise, for example, if solution electrolytes are required for the structural integrity of CFTR. An effect on CFTR functions within ABC transporters (1), many of which transport lipids, bile salts, conjugates with glutathione or sulfates, nucleoside-based conjugates (32, 33), or the bath solution.

Alternatively, electrolytes may be required to allow CFTR to complete a hydrolysis cycle because they participate in that process directly, for example, by being a substrate for transport, or a necessary cofactor in transport. This possibility is made less plausible by the lack of any evidence that CFTR is a transporter and by the lack of specificity in the ion species that can support activity. However, other members of the ABC transport family, such as MDR1 (P-glycoprotein), transport an extremely diverse set of substrates, which have little in common except hydrophobicity (42). That the dependence of CFTR gating on cytosolic electrolytes is most similar to the dependence on ATP itself raises the possibility that CFTR might retain either a transport function or at least components of transport that might now serve to regulate gating.

Although we found no prior example of an electrolyte effect on “typical” channels, transporters with requirements for specific electrolytes are commonplace, and some electrolyte-dependent transporters also function as ion channels (43, 44). CFTR is the only known ion channel among at least 39 human ABC transporters (1), many of which transport lipids, bile salts, conjugates with glutathione or sulfates, nucleoside-based compounds, or simply hydrophobic compounds (1). ABC1, which transports cholesterol (45), also appears to be a cAMP-dependent and sulfonilurea-sensitive anion transporter, although it is not itself a channel (46). The presence of multiple functions within ABC transporters and the coexistence of channel and transport functions in other proteins suggest that a possible transport function for CFTR should not be dismissed. If CFTR gating depends on the transport of a substrate, that substrate would need to be present within the bilayer (47) or the bath solution.

Regardless of the merits of these speculations, the discovery that CFTR gating depends on the electrolyte concentration of the cytosol provides a new tool for probing gating and ATPase hydrolysis cycles in CFTR and perhaps in other ABC transporters.

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Cystic Fibrosis Transmembrane Conductance Regulator Gating Requires Cytosolic Electrolytes
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