Cystic Fibrosis Transmembrane Conductance Regulator Inverts Protein Kinase A-mediated Regulation of Epithelial Sodium Channel Single Channel Kinetics*

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Abnormal regulation of ion channels by members of the ABC transport protein superfamily has been implicated in hyperinsulinemic hypoglycemia and in excessive Na⁺ absorption by airway epithelia in cystic fibrosis (CF). How ABC proteins regulate ion conductances is unknown, but must generally involve either the number or activity of specific ion channels. Here we report that the cystic fibrosis transmembrane conductance regulator (CFTR), which is defective in CF, reverses the regulation of the activity of single epithelial sodium channels (ENaC) by cAMP. ENaC expressed alone in fibroblasts responded to activation of cAMP-dependent protein kinase with increased open probability (Pₒ) and mean open time, whereas ENaC co-expressed with CFTR exhibited decreased Pₒ and mean open time under conditions optimal for PKA-mediated protein phosphorylation. Thus, CFTR regulates ENaC at the level of single channel gating, by switching the response of single channel Pₒ to cAMP from an increase to a decrease.

Recent studies (1, 2) have identified ENaC as the channel that mediates amiloride sensitive Na⁺ absorption in mammalian airways. In cystic fibrosis (CF), ENaC-mediated Na⁺ absorption is increased 200–300% in airway epithelia and, abnormally, further stimulated by raising intracellular cAMP (3). Because most CF mutations result in little if any functional CFTR in the apical cell membrane of affected epithelia (4), we inferred that normal CFTR must either down-regulate the number of active Na⁺ channels or decrease the activity of individual Na⁺ channels. In the present study we have studied the effects of cAMP-dependent protein-phosphorylating conditions on the single channel kinetics of ENaC expressed alone or together with CFTR in NIH 3T3 fibroblasts.

EXPERIMENTAL PROCEDURES

α-, β-, and γ-ENaC subunits were stably expressed in NIH 3T3 cell lines that had been previously transduced with a truncated (inactive) interleukin-2 receptor (ENaC alone cells) or with human CFTR (ENaC + CFTR cells) (5). ENaC-mediated single channel currents were recorded from cell attached and excised membrane patches as described in the figure legends.

RESULTS

The single channel conductance (4–5 picosiemens) of ENaC expressed in NIH 3T3 fibroblasts, as well as cation selectivity (Li⁺ > Na⁺ > K⁺), amiloride inhibition (Ki ≈ 0.3 μm) and the slow gating pattern (MT ≈ 1 s), are similar to what has been reported for the cloned channel expressed in oocytes (6, 7) and for endogenously expressed ENaC in rat cortical collecting tubule (8) or A6 cells (9) (Fig. 1). These similar results in very different cells suggest that cell specific cytoskeletal or other elements are not critical determinants of the basic biophysical characteristics of ENaC. The basal conductance and amiloride sensitivity of ENaC were not affected by co-expression with CFTR (Fig. 1).

ENaC present in excised membrane patches exhibited a variable degree of rundown following excision. Rundown was partially reversed (Fig. 2A, panel i) or prevented (Fig. 2A, panel ii) by exposure of the cytoplasmic surface to PKA catalytic subunit and 2 mM ATP (CS + ATP). Fig. 2A, panel iii, summarizes the results from both paradigms, revealing positive regulation of ENaC activity by PKA. One explanation for a range of basal activity, for rundown following excision, and for variable degree of activation by CS + ATP is that the resting phosphorylation state differs from patch to patch. Moreover, it seemed possible that water-soluble reagents, such as PKA catalytic subunit, might have poor access to hydrophobic compartments within the membrane patch. We tested these possibilities with a specific peptide inhibitor of PKA (mPKI) that had been modified by myristoylation to promote its association with biologic membranes (10, 11). mPKI was effective in (6/6) inside out membrane patches, reversing the effects of exogenous CS + ATP (Fig. 2A) by inhibiting Pₒ (Fig. 2A, panel iii) and MOT (not shown) to levels lower than “basal.” This observation suggests that the level of basal phosphorylation in the system influences the gating of ENaC in the absence of external manipulation.

The presence of CFTR caused a dramatic change in the regulation of ENaC in excised patches by CS + ATP. Whereas the gating and rundown of ENaC in patches excised from CFTR expressing cells were not obviously abnormal under nonstimulated conditions, exposure to CS + ATP routinely inhibited ENaC activity in two different paradigms (Fig. 2B). First, in 4/5 excised inside out patches, CS + ATP decreased Pₒ (Fig. 2B, panel i). Second, ENaC in 5/5 patches excised from CFTR expressing cells directly into CS + ATP demonstrated low Pₒ (Fig. 2B, panel ii) and MOT (not shown). mPKI further decreased Pₒ of ENaC co-expressed with CFTR (Fig. 2B, panels i and iii). Fig. 2B, panel iii, summarizes the very different pattern of regulation of ENaC by PKA in the presence of CFTR.
mediated regulation of whole cell amiloride-sensitive Na\(^{+}\) ATP in excised patches, strongly indicates that the CFTR-C ward (inward) currents represent cations leaving the pipette.

versus D

P

1

Instruments). Selected recorded currents were filtered (50 Hz, Ithaco) while recording active channels by diluting the bath by \(\frac{1}{2}\) with 200% of

bath. mPKI refers to the

1

paradigm carried out on a patch excised from a ENaC cell.

CFTR cell.

Effect of CFTR on regulation of ENaC by PKA in excised patches. A: panel i, current recorded from an inside out patch of ENaC only cell, starting just after excision. "c" indicates all channels closed. The probability of one channel being open decreased from 0.72 in the first 60 s following excision to 0.42 in the 60 s before addition of CS + ATP (rundown) and increased during exposure to CS + ATP to 0.65 in the last 60 s before addition of mPKI. mPKI completely inhibited ENaC. Panel i is representative of six experiments carried out with this paradigm. Panel ii, experiment illustrating the excision of an ENaC only cell attached patch directly into bath solution containing CS + ATP. Up to six ENaC remain active until exposure to mPKI by addition to the bath. Panel ii is representative of five patches excised into CS + ATP. Panel iii, summary of \(P_o\) calculated from data recorded (minimum duration of 60 s) from inside out patches exposed to different bath solutions. Basal (\(n = 11\)) includes the six patches from panel i and five patches studied under basal conditions only, CS + ATP (\(n = 11\)) includes all patches from panels i and ii; and mPKI (\(n = 6\)) includes five patches from panel i and one patch from panel ii. *, different from basal by unpaired t test, \(p < 0.05\). **, different from CS + ATP by unpaired t test, \(p < 0.01\). B: panel i, similar experiment as in A (panel i) but paradigm carried out on a patch excised from a ENaC + CFTR cell. Panel ii, effect of excision into CS + ATP on ENaC in a patch made from an ENaC + CFTR cell. Panel iii, summary of \(P_o\) of ENaC + CFTR patches, as described for A, panel ii. Basal, \(n = 10\), CS + ATP (\(n = 10\)), mPKI (\(n = 5\)). Methods: membrane patches were excised in the inside out mode. Basal refers to stationary channel activity following excision or just before exposure to CS and ATP. "CS + ATP" refers to the highest \(P_o\) observed during a minimal interval of 60 s in the period 3–10 min following exposure to 100 units/ml CS (Promega) + 2 mM ATP to the bath. mPKI refers to the \(P_o\) recorded in the period from 15 to 75 s following exposure to 1 \(\mu\)M mPKI (Biolmol) in the bath. \(P_o\) was determined from amplitude histograms. For multichannel patches, \(P_o\) was calculated and \(P_o\) derived assuming independent and equal gating of each channel and observation of maximal number of channels in the patch during recording.

not affect the number of ENaC channels observed per patch (2.17 ± 0.29 (\(n = 28\)) without CFTR and 2.29 ± 0.29 (\(n = 26\)) with CFTR). Second, the MOT of unambiguous single channel openings in excised, and cell-attached patches under optimal conditions of PKA activation were markedly decreased by the presence of CFTR (Fig. 4). Thus, CFTR negative regulation of ENAC can be explained by decreased activity of individual ENaC channels.

DISCUSSION

Our data reveal a surprisingly strong positive regulation of ENaCalone by PKA. The low \(P_o\) recorded in the presence of mPKI (Fig. 3) and the high \(P_o\) and long MOT measured during PKA activation (Fig. 4) indicate that increasing protein phosphorylation increased the time ENaC occupied a stable open conformation. This result differs from the cAMP-dependent increase of the number of endogenous amiloride sensitive Na\(^{+}\) channels seen in A6 epithelial cells (9), which are reported to regulate surface expression of transport elements by mem-
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**REFERENCES**

1. Burch, L., Talbot, C., Knowles, M. R., Canessa, C., Rossier, B., and Boucher, R. C. (1995) *Am. J. Physiol.* **269**, C511–C518
2. Hummler, E., Barker, P., Gatzy, J., Beermann, F., Verdumo, C., Schmidt, A., Boucher, R., and Rossier, B. C. (1996) *Nat. Genet.* **11**, 325–328
3. Boucher, R. C., Stutts, M. J., Knowles, M. R., Canetey, L., and Gatzy, J. T. (1996) *J. Clin. Invest.* **98**, 1245–1257
4. Collins, F. S. (1992) *Science* **256**, 774–779
5. Stutts, M. J., Canessa, C. M., Olsen, J. C., Hamrick, M., Cohn, J. A., Rossier, B. C., and Boucher, R. C. (1995) *Science* **269**, 847–850
6. Canessa, C. M., Horisberger, J., and Rossier, B. C. (1995) *Nature* **371**, 467–470
7. Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gauthier, S., Horisberger, J. D., and Rossier, B. C. (1994) *Nature* **367**, 463–467
8. Pacha, J., Prindt, G., Antonian, L., Sauerbier, B., and Palmer, L. G. (1993) *Am. J. Physiol.* **265**, C1071–C1084
9. Ismailov, I. I., McDuffie, J. H., and Benos, D. J. (1996) *J. Biol. Chem.* **271**, 2565–2571
10. Ismailov, I. I., Awaysa, M. S., Jovov, B., Berdevie, B. K., Fuller, C. M., Dedman, J. R., Keaetle, M. A., and Benos, D. J. (1996) *J. Biol. Chem.* **271**, 4725–4732

**Fig. 3. Effects of cAMP on open probability of ENaC studied on cell.** A, cell-attached patch of ENaC only expressing cell. Pipette current was recorded at 30 mV (Vpipette). Cell-permanently cAMP (cAMP) (500 μM) and forskolin (FSK; 10 μM) were added (as indicated by the arrow). The second and third traces were recorded 90 and 180 s later, respectively. For analysis, the P, during basal conditions (Basal, n = 8) and after stimulation (Stim, n = 8) were compared. (Histogram; p < 0.05, n = 8). B, effect of cAMP and forskolin (FSK) on ENaC activity in a cell-attached patch from an ENaC plus CFTR expressing cell. Analyzed as in A. (Histogram; n = 8 in each condition). Methods: cell-attached recordings were carried out under basal conditions (Basal, prior to addition) and stimulated conditions (Stim, 3–8 min following 500 μM cAMP and 10 μM forskolin), at −Vpipette of −20 to −40 mV. A minimum of 60 s of data was analyzed from each experiment. P, was determined as above.

**Fig. 4. CFTR alters cAMP regulation of ENaC kinetics (P₀ and MOT).** Excised inside-out patches or cell-attached patches that demonstrated only single ENaC during the entire experiment or patches with two channels that exhibited infrequent coincident openings were selected from the experiments presented in Figs. 2 and 3 to determine the effects of CFTR on ENaC gating in the presence of maximal PKA activity. Methods: P₀ was calculated as above, and lists of the durations of unambiguous openings were compiled from each experiment, with the events list feature of PCamps (Axon Instruments). Very long openings precluded sufficient observations for conventional analysis of the distribution of open time durations. Accordingly, the arithmetic average of all openings greater than 40 milliseconds was calculated as an estimate of mean open time (MOT), for each experiment (minimum 60 s or 40 openings analyzed). ENaC + CFTR (n = 7) different from ENaC (n = 9) by unpaired t analysis (p < 0.02).

Two consensus PKA phosphorylation sites, but these are not highly conserved across species (6, 7). Thus, PKA regulation of ENaC gating may well involve the phosphorylation and function of an additional protein or proteins, including cytoskeletal components such as actin (17). Cell-specific expression of these proteins could explain why fibroblasts reproduce the defect in CF airways better than oocytes (15).

In intact oocytes (15), or in ENaC reconstituted in lipid bilayers after expression in oocytes (16), the presence of CFTR decreased whole cell currents or single channel open probability. Thus, CFTR appears to exert a negative modulatory regulation of ENaC in several distinct cell types, including human airway epithelia, mouse fibroblasts, and amphibian oocytes.

The present findings help explain the long-standing observation that Na⁺ absorption across CF airway epithelia is increased and inappropriately further stimulated by cAMP (3). In CF airways, the abnormally high rate of basal Na⁺ absorption reflects the absence of negative regulation of ENaC by CFTR under basal phosphorylating conditions, and increased PKA activity leads only to further absorption. In contrast, CFTR function in normal airways converts the activation of PKA into a stimulus for both inhibition of ENaC-mediated Na⁺ absorption and stimulation of CFTR-mediated Cl⁻ secretion. Despite previous reports of abnormal regulation of Na⁺ channel activity in CF (18–20), this conclusion was in doubt until now, because PKA has been reported to regulate only the number of active amiloride-sensitive Na⁺ channels in A6 cells (9), and because another genetic disease associated with excessive Na⁺ reabsorption (Liddle’s syndrome) has been attributed solely to increased ENaC number (21). More recently, the mutations associated with Liddle’s syndrome have been shown to act predominantly by increased ENaC P₀ and MOT (22). This observation, coupled with the present results, make it clear that regulation of ENaC single channel kinetics is broadly implicated in the control of epithelial sodium absorption.

A general mechanism of regulation of ion channels by ABC proteins is yet to be identified (23), but it is clear that CFTR regulates ENaC at the level of single channel gating. This observation is an important consideration for understanding the mechanism by which ABC proteins, including not only CFTR but also SUR and MDR (23), can influence other ion channels. Potentially, ABC proteins regulate the activity of other ion channels through transported substrates, as proposed for CFTR-mediated ATP release (24, 25). Alternatively, ABC proteins may regulate the activity of other ion channels by direct or indirect protein-protein interactions.
17. Berdiev, B. K., Prat, A. G., Cantiello, H. F., Ausiello, D. A., Fuller, C. M., Jovov, B., Benos, D. J., and Ismailov, I. I. (1996) *J. Biol. Chem.* **271**, 17704–17710
18. Chinet, T. C., Fullton, J. M., Yankaskas, J. R., Boucher, R. C., and Stutts, M. J. (1993) *Am. J. Physiol.* **265**, C1050–C1060
19. Chinet, T. C., Fullton, J. M., Yankaskas, J. R., Boucher, R. C., and Stutts, M. J. (1994) *Am. J. Physiol.* **266**, C1061–C1068
20. Duszak, M., French, A. S., and Man, S. F. P. (1991) *Biomed. Res.* **12**, 17–23
21. Snyder, P. M., Price, M. P., McDonald, F. J., Adams, C. M., Volk, K. A., Zeiher, B. G., Stokes, J. B., and Welsh, M. J. (1995) *Cell* **83**, 969–978
22. Hansson, J. H., Schild, L., Lu, Y., Wilsen, T. A., Gautschi, I., Shinketsu, B., Nelson-Williams, C., Rossier, B. C., and Lifton, R. P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11495–11499
23. Higgins, C. F. (1995) *Cell* **82**, 693–696
24. Cantiello, H. F., Prat, A. G., Reisin, I. L., Ercole, L. B., Abraham, E. H., Amara, J. F., Gregory, R. J., and Ausiello, D. A. (1994) *J. Biol. Chem.* **269**, 11224–11232
25. Schwieber, E. M., Egan, M. E., Hwang, T., Fulmer, S. B., Allen, S. S., Cutting, G. R., and Guggino, W. B. (1995) *Cell* **81**, 1063–1073
26. Stutts, M. J., Gabriel, S. E., Olsen, J. C., Gatzy, J. T., O’Connell, T. L., Price, E. M., and Boucher, R. C. (1995) *J. Biol. Chem.* **268**, 20653–20658
27. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) *Pfluegers Arch.* **391**, 85–100
28. Hagiwara, T. (1996) *Cell* **85**, 1095–1098