preliminary to allow conclusive statistical analysis.

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9. The digests were fractionated on 0.8% agarose gels and transferred to nylon membranes for hybridization. The pure human samples contained 10 to 15 μg of DNA, whereas the pure yeast samples contained 0.1 to 0.2 μg; the mixed samples contained 10 to 15 μg of human and 0.1 to 0.2 μg of yeast DNA. Small differences in the electrophoretic mobilities of corresponding fragments detected in the human and yeast lanes of Fig. 3 are due to differences in the amounts of DNA loaded in the two cases. Because of the 200-fold disparity in the sizes of the yeast and human genomes, 50 ng of YAC-containing yeast DNA gives a hybridization signal comparable to that obtained from 10 μg of human DNA. When 10 to 15 μg of human DNA are mixed with 0.1 to 0.2 μg of yeast DNA the electrophoretic mobilities of the hybridizing bands are identical to those observed in the pure human samples, but the intensities of bands that contain YAC-derived fragments are enhanced (lanes 2 and 4 of all panels).
10. Methods of producing YAC clones from Eco RI partial digests of high molecular weight human DNA are described elsewhere (*1, 8*). Clones that were ura^+, trp^+, and lacked *ochre* suppression were grown to saturation in 1-ml cultures in the rich medium YPD [F. Sherman, G. R. Fink, J. B. Hicks, *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986), p. 163]. Samples were adjusted to a glycerol concentration of 20% and stored at −80°C in microtiter plates. A custom-fabricated, multiprong replicator was used to stamp the colony grids onto SUREBLOT nylon membranes (Oncor), which were then placed in contact with the ura^- trp^- medium $AHC^=$ [per liter: 1.7 g of yeast nitrogen base (Difco 0335-15), 5 g of $(NH_4)_2SO_4$, 10 g of casein hydrolysate (U.S. Biochemicals 12852), 20 g of glucose, and 20 mg of adenine (*p*H 5.8)] and incubated at 30°C. Cells were converted to spheroplasts and lysed by sequentially placing the nylon filters in contact with a series of reagent-saturated paper filters. In the order of use, the reagents and treatment times were as follows: lyticase mixture CDY [yeast lytic enzyme (2 mg/ml) (ICN 152270, >70,000 U/g), 1.0*M* sorbitol, 0.1*M* sodium citrate, 0.05*M* EDTA, and 0.015*M* dithiothreitol (*p*H 7)], overnight; 10% SDS, 5 min; 0.5*M* NaOH, 10 min; 2× saline sodium citrate, *p*H 7, and 0.2*M* tris HCl, *p*H 7.5, three times for 5 min each. The lyticase reaction was carried out at 30°C, and the remaining treatments were at room temperature. Filters were air-dried. Labeling of probes, hybridization, and autoradiography were performed in accordance with standard methods [A. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983); T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)].
11. Methods of preparing high molecular weight yeast DNA in agarose plugs have been described [D. C. Schwartz and C. R. Cantor, *Cell* **37**, 67 (1984); G. F. Carle and M. V. Olson, *Methods Enzymol.* **155**, 468 (1987)]. For electrophoresis, the contour-clamped homogeneous electric field system was used [G. Chu, D. Vollrath, R. W. Davis, *Science* **234**, 1582 (1986)]. The electric field was 6.0 V/cm, the temperature 12° to 14°C, the running time 22 to 24 hours, and the running buffer was half-strength tris borate (Carle and Olson, above). For the gel in Fig. 2A, a ramped switching interval starting at 35 s and ending at 70 s was used, and for the gel in Fig. 2B, a constant switching interval of 70 s was used. The former conditions provide better resolution in the region most relevant for the analysis of typical YACs. The probe for factor IX was prepared by gel purifying the 1.0-kb Eco RI–Hind III insert in a factor IX cDNA clone whose insert spans nucleotides 1–1029, as numbered by M. Jaye *et al.* [*Nucleic Acids Res.* **11**, 2325 (1983)], and which recognizes all eight of the factor IX exons; the hybridization of this probe with Taq I–digested human DNA has been described [G. Camerino, M. G. Mattei, J. F. Mattei, M. Jaye, J. L. Mandel, *Nature* **306**, 701 (1983)]. The probe for PAI-2 was prepared by gel purifying the 1.9-kb full-length cDNA fragment described in (*5*), which had been subcloned into the Eco RI site of pGEM-4.

12. We acknowledge R. Rothstein for providing an unpublished yeast colony-screening protocol. M. A. Hutchinson, P. Dang, S. C. Silver, and M. Lackey provided technical assistance. J. L. Mandel provided the factor IX cDNA clone and J. E. Sadler provided the PAI-2 cDNA clone. R. Waterston made helpful suggestions on the manuscript. Supported by a grant from the James S. McDonnell Foundation to the Center for Genetics in Medicine, Washington University, St. Louis, MO, and by NIH grant GM40606. G.A.S. was supported by a National Research Service Award (HD07271) from the National Institute of Child Health and Human Development.

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Cl^- Channels in CF: Lack of Activation by Protein Kinase C and cAMP-Dependent Protein Kinase

TZYH-CHANG HWANG, LUO LU, PAMELA L. ZEITLIN, DIETER C. GRUENERT, RICHARD HUGANIR, WILLIAM B. GUGGINO*

Secretory chloride channels can be activated by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase in normal airway epithelial cells but not in cells from individuals with cystic fibrosis (CF). In excised, inside-out patches of apical membrane of normal human airway cells and airway cells from three patients with CF, the chloride channels exhibited a characteristic outwardly rectifying current-voltage relation and depolarization-induced activation. Channels from normal tissues were activated by both cAMP-dependent protein kinase and protein kinase C. However, chloride channels from CF patients could not be activated by either kinase. Thus, gating of normal epithelial chloride channels is regulated by both cAMP-dependent protein kinase and protein kinase C, and regulation by both kinases is defective in CF.

THE APICAL Cl^- CONDUCTANCE OF airway epithelia controls transepithelial Cl^- secretion. The magnitude of Cl^- secretion, which is regulated by several secretagogues, determines the quantity of respiratory tract fluid (*1*). The apical Cl^- conductance of epithelia from patients with CF is abnormally low because of an inability of β-adrenergic agonists to increase the magnitude of the apical Cl^- conductance (*2*). This abnormality contributes to the production of thick mucous in CF patients.

Airway epithelia contain outwardly rectifying Cl^- channels that are activated through cAMP-dependent pathways (*3*). The purified catalytic subunit of cAMP-

T.-C. Hwang, L. Lu, P. L. Zeitlin, W. B. Guggino, Department of Physiology, The Johns Hopkins University, Baltimore, MD 21205.
D. C. Gruenert, Cardiovascular Research Institute, University of California, San Francisco, CA 94143.
R. Huganir, Howard Hughes Medical Institute, and Department of Neuroscience, The Johns Hopkins University, Baltimore, MD 21205.

*To whom correspondence should be addressed.

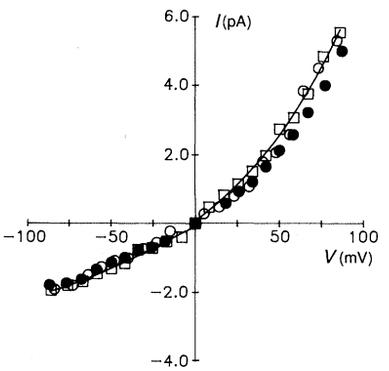

Fig. 1. Current-voltage relations (*I-V*) of Cl^- channels activated by PKC in normal human fetal tracheal cells and by depolarizing voltages both in adult normal and CF airway cells. The number of channels in a patch varied from one to four. Data were recorded by an EPC-7 patch-clamp amplifier (List Darmstadt, Federal Republic of Germany), filtered at 1 kHz (Frequency Devices, Haverhill, MA) digitized with a pulse-code modulator (Japanese Victor Corporation), and stored on video cassette recorder (Japanese Victor Corporation). Data were analyzed on a PDP 11/23 computer (Digital Equipment Corporation). The bath solution contained 150 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* EGTA, 5 m*M* Hepes, and 0.5 m*M* $CaCl_2$ (free Ca^{2+} was 1.2 μ*M*). The pipet contained 150 m*M* NaCl, 2 m*M* $CaCl_2$, and 5 m*M* Hepes. The *p*H of both solutions was adjusted to 7.3 with tris base. With 300 m*M* Cl^- in the bath, the reversal potential (15 mV) was close to that expected for the Cl^- concentration gradient (16.7 mV). Graph was drawn by Sigmaplot Version 3.1 (Jandel Scientific). ●, human fetus; ○, human adult; □, human CF.

dependent protein kinase (PKA) can directly activate normal Cl^- channels in excised patches, whereas Cl^- channels from CF patients that have the same outward rectification and single channel conductance fail to respond to the kinase (*4*, *5*). This suggests that defective phosphorylation may be responsible for the lack of β-adrenergic agonist stimulation of Cl^- secretion in CF tissues.

Epithelial Cl^- secretions can also be regulated by cAMP-independent pathways. For example, both the Ca^{2+} ionophore A23187 and prostaglandin F2α stimulate Cl^- secretion without inducing measurable changes in intracellular cAMP concentration (*6*). Furthermore, the phorbol ester phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, also increases Cl^- secretion without causing a rise in intracellular cAMP, although the actual first messenger of PKC stimulation is not known (*7*, *8*). We have used the patch-clamp technique to determine whether purified PKC and PKA can regulate Cl^- channels in normal adult and fetal airway epithelia (*9*) and whether regulation by both kinases is defective in CF patients.

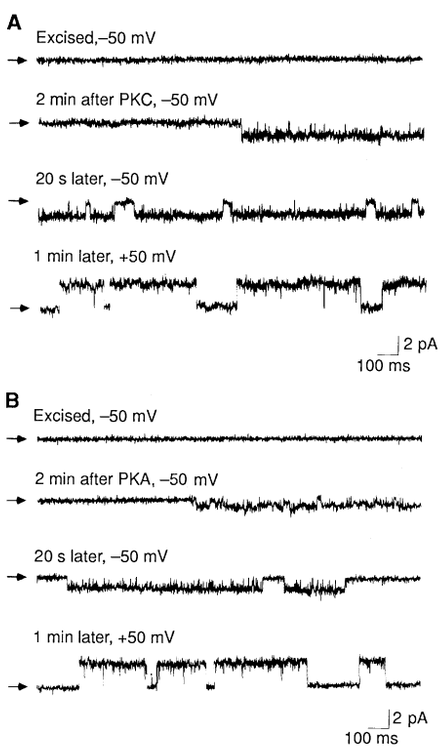

Fig. 2. Activation of fetal epithelial Cl^- channels by PKC and PKA. Excised membrane patches from human fetal tracheal cells were held at −50 mV for 2 min before PKC (**A**) or PKA (**B**) and cofactors, DiC_8 (Serdary, Ontario, Canada) and Mg-ATP (Sigma) for PKC and only Mg-ATP for PKA, were added to the solution facing the cytoplasmic side of the membrane. In six experiments, Walsh inhibitor peptide, a specific inhibitor for PKA, was added with PKC. Channel activity was monitored for another 8 min. PKC and PKA were prepared as described (*18*). Final concentrations of chemicals and enzymes were 1 m*M* ATP, 1 μ*M* DiC_8, 2 μ*M* Walsh inhibitor, 10 n*M* PKC, and 50 n*M* PKA. Arrows indicate the closed state.

Excised patches of cell membrane from normal fetal and adult and CF adult airways displayed no channel activity until they were depolarized to between +50 and +120 mV (all voltages are referenced to a bath ground). Voltage-activated channels had an outwardly rectifying current-voltage (*I-V*) relation with a reversal potential in asymmetrical solutions consistent with Cl^- channels (Fig. 1). The electrophysiological properties were identical to those reported previously for secretory Cl^- channels (*3–5*). Thus, fetal and adult airway cells contain the same type of secretory Cl^- channels.

To determine whether secretory Cl^- channels are regulated by both PKC and PKA, we maintained excised patches first at −50 mV to avoid voltage activation. After an initial period of 2 min to verify the absence of spontaneous channel activity, PKC or PKA plus cofactors [adenosine triphosphate (ATP), and dioctanoylglycerol (the diacylglycerol analog DiC_8)] was added to the bath. After channel activity was observed, the voltage was changed to values between +50 and +87 mV to verify that the kinase-activated channels were epithelial Cl^- channels. No spontaneous channel activity was seen at negative voltages for at least 8 min with buffer containing only the cofactors (0/10); the presence of Cl^- channels in these patches was verified by depolarization. In normal fetal and adult airway cells, Cl^- channel activity was evoked within 7 min of the addition of PKC or PKA (Table 1 and Fig. 2). The time courses of activation by PKC and PKA were very similar. PKC did not activate Cl^- channels (0/4) in the presence of the PKC inhibitor staurosporine (*10*). Staurosporine by itself did not activate Cl^- channels (0/3) or inhibit subsequent voltage activation. In contrast, PKC activated channels in the presence of the Walsh PKA inhibitor (*11*). The concentration of the PKA inhibitor was sufficient to completely block the activation of Cl^- channels by PKA (0/3). Therefore, PKC and PKA can directly activate Cl^- channels. The *I-V* characteristics of PKC- and PKA-activated Cl^- channels were identical to those activated by depolarizing voltages (Fig. 1).

Table 1. Protein kinase activation of Cl^- channels. PKA, 50 n*M*; PKC, 10 n*M*. Normal adult human epithelia include primary cultures of nasal scrapings and tracheal epithelial primary cultures. Fetal human tracheal cells were transformed with an origin of replication-defective SV40 plasmid (*17*). The adult CF group includes primary cultures of nasal polyps from two CF patients, and both primary and SV40–infected cultures of CF bronchial epithelia from one CF patient. Data are expressed as "number of patches activated by protein kinases/number of patches containing Cl^- channels." In the adult CF cells, the presence of channels was confirmed by voltage activation. Experiments in which channels were not observed after kinase treatment and after depolarization were not included (ten with fetal cells, three with normal adult cells, and five in CF cells).

Source	PKA	PKC	PKA + PKC
Fetal tracheal epithelia	6/7	7/8	
Adult airway epithelia		3/3	
Adult CF airway epithelia	0/9	0/7	0/3

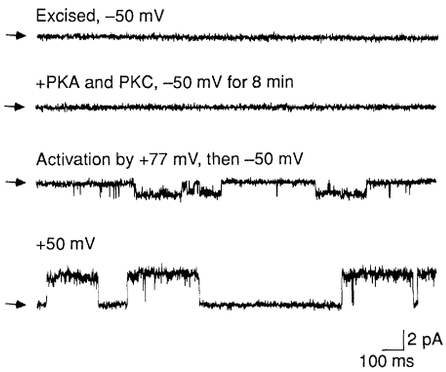

Fig. 3. Failure of both PKC and PKA to activate a Cl^- channel in a CF cell. The experimental conditions were as in Fig. 2.

To ascertain if PKC activation of Cl^- channels is defective in CF, we applied both PKC and PKA to patches of membrane excised from bronchus and nasal polyp cells from patients with CF. In CF cells, the catalytic subunit of PKA was not able to activate the channels, as has been reported (*4*, *5*). In addition, we found that PKC alone or in combination with PKA failed to open channels in membrane patches from three different CF patients (Table 1 and Fig. 3). In these experiments, the presence of Cl^- channels was verified by depolarization activation (Fig. 3).

Phosphorylation sites are present on several different types of ion channels and are important in channel regulation (*12*). For example, both PKA and PKC phosphorylate the nicotinic acetylcholine receptor (*13*) and the dihydropyridine receptor (*14*) of the Ca^{2+} channel in skeletal muscle. Phosphorylation by both kinases accelerates the rate of desensitization of the nicotinic receptor (*13*). In cardiac cells both L-type Ca^{2+} (*15*) and delayed rectifier K^+ channels (*16*) are activated by PKC and PKA phosphorylation. PKA also activates epithelial Cl^- channels (*4*, *5*). Our work suggests the existence of either multiple phosphorylation sites or a single site that can be phosphory-

lated either by PKC or PKA. Thus, several hormonal signals operating via cAMP-dependent and cAMP-independent pathways lead separately to stimulation of Cl^- channel activity.

Both PKC and PKA are unable to activate epithelial Cl^- channels from CF patients. Our data suggest that both kinases act on a common pathway directly involved in Cl^- channel gating. If one assumes that CF arises from a single mutation in the CF gene, then this mutation could cause a Cl^- channel defect either at a common PKC and PKA phosphorylation site or at a specific domain that couples separate phosphorylation sites to channel activation.

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9. Cells were grown in flasks coated with a mixture containing distilled water, fibronectin (10 μg/ml), collagen (6 mg/ml), and bovine serum albumin (100 μg/ml) and maintained at 37°C in 5% CO_2 and 95% air. Normal fetal, normal adult, CF nasal polyps, and SV40–infected CF bronchial cells were grown in LHC-8 medium (Biofluids, Rockville, MD) with penicillin (100 unit/ml) and streptomycin (100 μg/ml). For patch clamping, cells were passed onto cover glasses coated as above. Primary bronchial CF cells were cultured in airway medium [P. L. Zeitlin, G. M. Loughlin, W. B. Guggino, *Am. J. Physiol.* **254**, C691 (1988)] containing endothelial cell growth supplement (25 μg/ml), T_3 (50 p*M*), imipenem (200 μg/ml), and tobramycin (80 μg/ml).
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19. We thank E. Rohland for technical assistance with cell cultures, V. Rothman for technical assistance in preparing PKC, and D. Raben for many helpful discussions. We thank the patients and families, and surgeons who helped us obtain CF tissue. Supported by NIH grant R01-HL 40178 and Cystic Fibrosis Foundation grant Z028-8 to W.B.G.; NIH Clinical Investigator Award 1-K08-HL02188 to P.Z.; NIH grant R01-DK 39619 and Cystic Fibrosis Foundation Research Development grant to D.C.G.

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Regulation of Chloride Channels by Protein Kinase C in Normal and Cystic Fibrosis Airway Epithelia

MING LI, JOHN D. MCCANN, MATTHEW P. ANDERSON, JOHN P. CLANCY, CAROLE M. LIEDTKE, ANGUS C. NAIRN, PAUL GREENGARD, MICHAEL J. WELSH*

Apical membrane chloride channels control chloride secretion by airway epithelial cells. Defective regulation of these channels is a prominent characteristic of cystic fibrosis. In normal intact cells, activation of protein kinase C (PKC) by phorbol ester either stimulated or inhibited chloride secretion, depending on the physiological status of the cell. In cell-free membrane patches, PKC also had a dual effect: at a high calcium concentration, PKC inactivated chloride channels; at a low calcium concentration, PKC activated chloride channels. In cystic fibrosis cells, PKC-dependent channel inactivation was normal, but activation was defective. Thus it appears that PKC phosphorylates and regulates two different sites on the channel or on an associated membrane protein, one of which is defective in cystic fibrosis.

CHLORIDE SECRETION BY AIRWAY epithelium is controlled in part by Cl^- channels in the apical membrane of the cell (*1*). These channels are in turn regulated by adenosine 3′,5′-cyclic monophosphate (cAMP)–dependent protein kinase (PKA), which phosphorylates the Cl^- channel or an associated protein, thereby activating (*2*) the channel (*3*). In cystic fibrosis (CF), regulation of this channel is defective: PKA does not activate Cl^- channels, although these channels are present in the membrane and can be activated by depolarization after membrane patches are excised from the cell (*3, 4*). Defective regulation of Cl^- channels in epithelium from patients with CF may contribute to the pathophysiology of this genetic disease (*5*).

PKC is important in regulating secretion (*6*), and it controls ion channels in several cell types (*7*). In airway epithelia, two Cl^- secretagogues, isoproterenol and bradyki-

M. Li, J. D. McCann, M. P. Anderson, J. P. Clancy, M. J. Welsh, Laboratory of Membrane Transport and Pulmonary Division, Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA 52242.

C. M. Liedtke, Cystic Fibrosis Center, Departments of Pediatrics and Physiology and Biophysics, School of Medicine, Case Western Reserve, Cleveland, OH 44106.

A. C. Nairn and P. Greengard, Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York, NY 10021.

*To whom correspondence should be addressed.

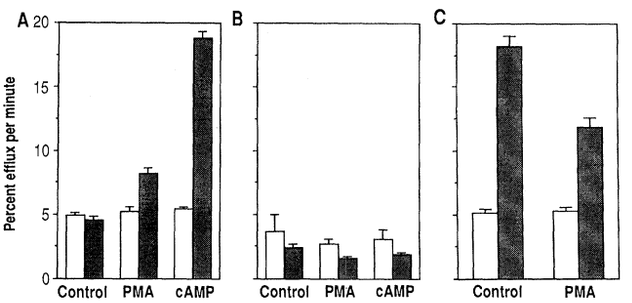

Fig. 1. Effect of PMA and cAMP on $^{125}I^-$ efflux. (**A**) Efflux was measured under control conditions (Control) or with addition of PMA (PMA) (100 n*M*) or 10 μ*M* forskolin plus 500 μ*M* IBMX (cAMP) (*9*). Data are mean ± SEM ($n = 11$ cover slips from three experiments); both PMA and cAMP increased efflux ($P < 0.05$). (**B**) Efflux in CF cells; conditions are the same as in Fig. 1A ($n = 4$ cover slips from one experiment). (**C**) Effect of PMA on the response to cAMP. Cells were exposed to PMA (100 n*M*) or vehicle (Control) for 10 min before addition of 10 μ*M* forskolin plus 500 μ*M* IBMX (cAMP) ($n = 10$ cover slips from three experiments). The cAMP-induced increase in efflux was less in the presence of PMA ($P < 0.05$). Values for all efflux rates were obtained 2 to 5 min after addition of agonists when efflux rate was greatest. Forskolin and IBMX produced comparable increases in cAMP in the presence and absence of PMA (*10*). In (C) baseline efflux rate was similar in the control and PMA groups because the PMA-stimulated increase in $^{125}I^-$ efflux was transient and had returned to baseline values 10 min after addition of PMA. □, Baseline; ■, intervention.