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


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**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-


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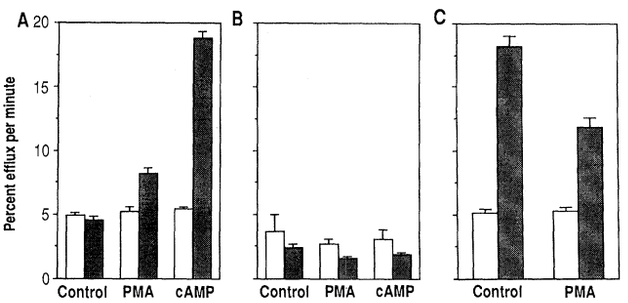


**Fig. 1.** Effect of PMA and cAMP on $^{125}I^-$ efflux. (**A**) Efflux was measured under control conditions (Control) or with addition of PMA (PMA) (100 n*M*) or 10 μ*M* forskolin plus 500 μ*M* IBMX (cAMP) (*9*). Data are mean ± SEM ($n = 11$ cover slips from three experiments); both PMA and cAMP 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.

nin, increase the cellular mass of diacylglycerol (*8*), an activator of PKC, suggesting that receptors in these cells can mediate activation of PKC. Therefore, we examined regulation of the $Cl^-$ channel by PKC.

To assess the effect of PKC on $Cl^-$ channels in the intact cell, we used phorbol 12-myristate 13-acetate (PMA), which is membrane-permeant, to activate PKC and measured $^{125}I^-$ efflux as an index of $Cl^-$ channel activation (*9*). PMA increased $^{125}I^-$ efflux (Fig. 1A), but the degree of stimulation was smaller than that produced by forskolin (10 $\mu M$) plus 3-isobutyl-1-methyl-xanthine (IBMX) (500 $\mu M$) [a treatment that increases cellular cAMP (*10*)].

In contrast, addition of PMA before forskolin plus IBMX attenuated cAMP-induced $^{125}I^-$ efflux (Fig. 1C). These results suggest that PKC can either stimulate or inhibit the $Cl^-$ channel, depending on the physiological status of the cell. They are also consistent with studies showing that PMA partially stimulated $Cl^-$ secretion, but also inhibited cAMP-induced $Cl^-$ secretion (*11*).

To assess more directly the effect of PKC on the $Cl^-$ channel, we used the single-channel patch-clamp technique to obtain cell-free, inside-out membrane patches from primary cultures of human and canine airway epithelia. PKC-dependent phosphorylation requires adenosine triphosphate (ATP), phosphatidylserine, and a tumor-promoting phorbol ester or diacylglycerol (*6*). Therefore we added PKC (*12*), ATP (1 m$M$), and dioctanoylglycerol (DiC8) (1 $\mu$g/ml), and the cell membrane served as the source of phospholipid.

At a low $Ca^{2+}$ concentration (<10 n$M$), PKC activated $Cl^-$ channels (Fig. 2A and Table 1). Activation occurred an average of 126 s after addition of PKC, DiC8, and ATP (maximum time, 210 s). In contrast, channels did not activate during 8 min of observation in paired patches not exposed to PKC (Table 1). Activation required the presence of PKC, ATP, and either DiC8 or PMA; addition of only two of the reagents was insufficient (*13*). In some patches PKC, ATP, and DiC8 did not activate a channel within 8 min of addition, even though subsequent depolarization (*3*) showed a channel present in the patch (Table 1).

When we increased internal $Ca^{2+}$ to >10 $\mu M$, the channel that had been activated by PKC at low $Ca^{2+}$ inactivated (Fig. 2A); in two of three other patches that could be adequately evaluated, increasing $Ca^{2+}$ concentration inactivated channels in an average of 25 s, indicating that at high $Ca^{2+}$, PKC had an effect opposite to that observed at low $Ca^{2+}$.

At high $Ca^{2+}$ concentration (1 $\mu M$), PKC also prevented voltage-dependent channel activation (Table 2A). In control patches, no channels activated during the 6 min intervention period, but subsequent depolarization (*3*) showed that most of the patches contained $Cl^-$ channels. In paired experiments, we added PKC, PMA (100 n$M$), and ATP to the internal surface of the patch; in contrast to results obtained at low $Ca^{2+}$ concentration, channels were not activated. In fact, PKC prevented subsequent channel activation in response to large membrane depolarization.

At high $Ca^{2+}$ concentration, PKC inactivated $Cl^-$ channels that had previously been

**Table 1.** Activation of $Cl^-$ channels by PKC or depolarization at low $Ca^{2+}$ concentrations. Voltage was −40 mV throughout. Data are from two groups of studies, each with its own controls. Patches were from 30 dog and 20 human cells. The internal (cytosolic) surface was exposed to purified PKC (*12*), ATP (1 m$M$), and either DiC8 (1 $\mu$g/ml) or PMA (100 n$M$), as indicated. If no channel activated during 8 min, the membrane was progressively depolarized (*3*) to voltages up to +140 mV. The number of channels activated during the intervention period was different for PKC and control groups ($P < 0.002$ by $\chi^2$ analysis).

| Intervention | $Ca^{2+}$ | Total patches | Patches with channel activated during: | | Blank patches |
|---|---|---|---|---|---|
| | | | Intervention | Depolarization | |
| Control | <10 n$M$ | 15 | 0 | 11 | 4 |
| PKC, DiC8, ATP | <10 n$M$ | 23 | 9 | 5 | 9 |
| Control | <10 n$M$ | 5 | 0 | 5 | 0 |
| PKC, PMA, ATP | <10 n$M$ | 7 | 5 | 0 | 2 |

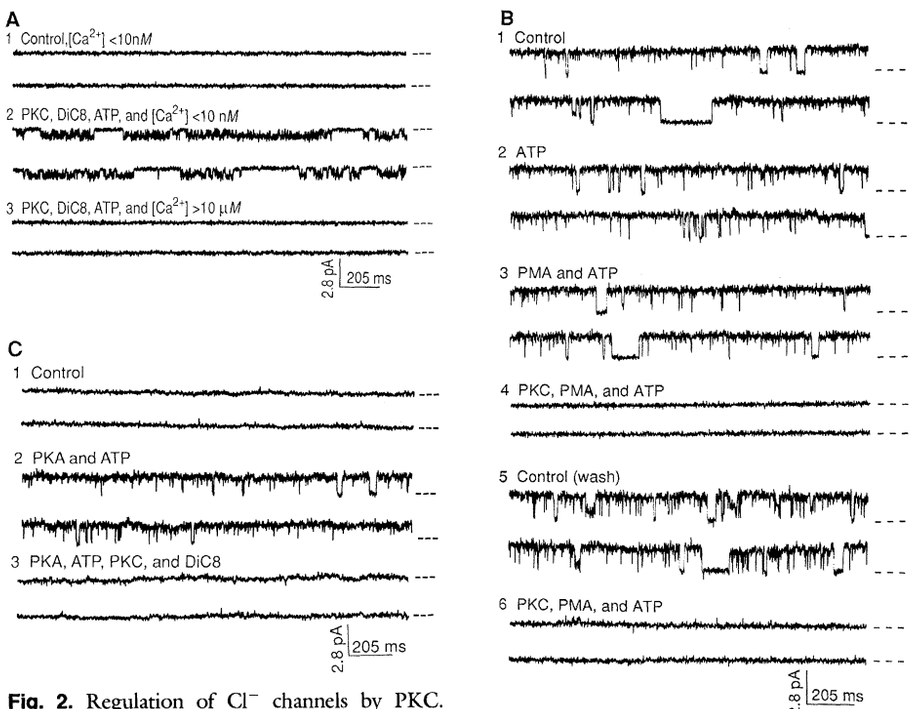


**Fig. 2.** Regulation of $Cl^-$ channels by PKC. Tracings are examples from excised, inside-out patches from canine (A) or normal human (B and C) airway epithelial cells (*21*). Inward current is shown as a downward deflection (A); outward current is shown as an upward deflection (B and C); the current level when channels are closed is shown by the dashed line. (**A**) PKC activation of a $Cl^-$ channel at low $Ca^{2+}$ (<10 n$M$). Membrane voltage was −40 mV throughout. (1) Control; (2) PKC, DiC8 (1 $\mu$g/ml), and ATP (1 m$M$) were added to the internal solution, and the channel activated 132 s later; (3) internal $Ca^{2+}$ was increased to >10 $\mu M$ by addition of $CaCl_2$ to the bath and the channel inactivated 52 s later. (**B**) PKC inactivation of a depolarization-activated $Cl^-$ channel at 1 $\mu M$ $Ca^{2+}$. Holding voltage was −40 mV and tracings were obtained at +40 mV. After channel was activated by depolarization, recordings were made under the following conditions: (1) Control, no additions (probability of the channel being in the open state ($P_o$) = 0.80); (2) ATP (1 m$M$) ($P_o$ = 0.89); (3) PMA (100 n$M$) and ATP ($P_o$ = 0.73); (4) PKC, PMA, and ATP ($P_o$ = 0); (5) control conditions after removal of PKC, PMA, and ATP from the internal solution ($P_o$ = 0.71); (6) readdition of PKC, PMA, and ATP ($P_o$ = 0). (**C**) PKC inactivation of a PKA-activated $Cl^-$ channel at high (1 $\mu M$) $Ca^{2+}$. Voltage conditions as in (B). (1) Control; (2) catalytic subunit of PKA and ATP (1 m$M$) added as described in (*3*); (3) PKA, ATP, PKC, and DiC8 (1 $\mu$g/ml).

activated by depolarization (Fig. 2B and Table 2B). Addition of ATP and then PMA to a depolarization-activated $Cl^-$ channel (Fig. 2B) did not alter channel kinetics. However, on addition of PKC, the channel inactivated. In control patches not exposed to PKC, $Cl^-$ channels remained in the activated state for at least 10 min, indicating that inactivation was not a random event. When internal $Ca^{2+}$ was <10 n*M*, PKC, DiC8, and ATP did not inactivate depolarization-activated channels. However when we subsequently increased internal $Ca^{2+}$ to >10 μ*M* in those same patches, channels inactivated.

At an estimated $Ca^{2+}$ concentration of 150 n*M*, we found variable effects of PKC, although it usually inactivated channels. In one series of experiments, depolarization failed to activate a channel in seven patches; in parallel controls, depolarization activated channels in two of four patches not exposed to PKC; PKC inactivated four depolarization-activated channels; and PKC activated one channel that was subsequently inactivated by increasing $Ca^{2+}$ to 10 μ*M*.

In some cases inactivation in the presence of high $Ca^{2+}$ was reversible (Fig. 2B). We were able to evaluate reversibility in 16 patches. In 12 of those 16 patches (6 from canine and 6 from normal human cells), removal of the phosphorylation solution resulted in reactivation (average time, 3 min). Reversibility of PKC-induced inactivation suggests that a membrane-associated phosphatase has access to the channel in the membrane patch.

Each of three activators of PKC—PMA (100 n*M*), DiC8 (1 μg/ml), or diolein (40 μg/ml)—was able to support inactivation of channels (Table 2B). PKC partially purified from canine trachea (*12*) was also effective in four of seven cases. Inactivation required the combination of PKC, ATP, and PMA or a diacylglycerol; addition of one or of any two alone was insufficient (*14*). Complete channel inactivation occurred an average of 196 ± 58 s (mean ± SEM) after addition of PKC, ATP, and PMA or a diacylglycerol. In many cases the kinetic properties of channels were altered 10 to 60 s after addition and before complete inactivation.

The $^{125}I^-$ efflux studies (Fig. 1C) indicated an interaction between the effects of PKA and PKC. To further address this issue, we first activated a $Cl^-$ channel by phosphorylation with the catalytic subunit of PKA and ATP (Fig. 2C) (*3*) and then studied the effect of subsequent addition of PKC plus DiC8 at 1 μ*M* $Ca^{2+}$. In six patches, PKA activated $Cl^-$ channels (average time, 174 s) and subsequent addition of PKC plus DiC8 ($n = 3$) or PMA ($n = 3$) inactivated channels (average time, 108 s).

Since regulation of $Cl^-$ channels by PKA is defective in CF (*3, 4*), we asked whether regulation by PKC was also abnormal. Neither PMA nor cAMP increased $^{125}I^-$ efflux in intact CF cells (Fig. 1B). In excised patches, PKC at low $Ca^{2+}$ concentration failed to activate any channels even though patches did contain $Cl^-$ channels that could be activated by depolarization (Table 3A, compare with data from normal cells in Table 1). These results indicate that in CF, PKC fails to activate $Cl^-$ channels either in the cell or in cell-free membrane patches.

At high $Ca^{2+}$ concentrations (1 μ*M*), the combination of PKC, PMA, and ATP inactivated CF $Cl^-$ channels, as it had in normal cells (Table 3B, compare with results from normal cells in Table 2B). The effect of PKC

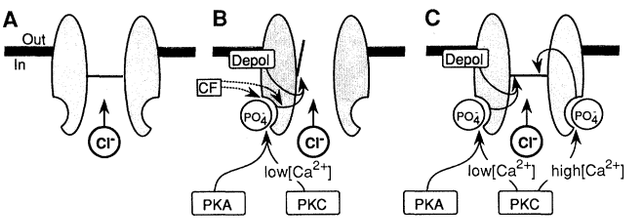


**Fig. 3.** Model of $Cl^-$ channel regulation. Inner and outer surfaces of the membrane are indicated in (A). Channel is defined as inactivated when the "gate" is closed (**A** and **C**) and activated when it is open (**B**) (*2*). The gate may involve different molecular steps. "Depol" refers to strong membrane depolarization (approximately +100 to +140 mV). At high $Ca^{2+}$ concentration, PKC may also phosphorylate the low $Ca^{2+}$ site. In this model and in the text, we refer to the channel as a single entity, but it may consist of multiple subunits and associated proteins.

**Table 2.** Inactivation of $Cl^-$ channels by PKC. (A) Patches were excised, holding voltage was −40 mV, and membrane voltage was stepped to +40 mV for 2 s every 10 s. Purified PKC, PMA (100 n*M*), and ATP were applied as indicated. If no channel activated in 6 min, we applied strong depolarization (up to 140 mV). (B) Patches were excised, channels were activated by strong depolarization (up to +140 mV), then holding voltage was −40 mV, and membrane voltage was stepped to +40 mV for 2 s every 10 s. Purified PKC, ATP, DiC8, PMA, and diolein were then added as indicated. In (A) the number of channels activated in the two conditions was different ($P < 0.001$ by $\chi^2$ analysis). In (B) the number of channels inactivated in the control and the low $Ca^{2+}$ groups were different from the other three groups ($P < 0.001$ by $\chi^2$ analysis). Data are from 38 dog and 25 human cells.

(A) *Prevention of depolarization-induced activation*

| Intervention | $Ca^{2+}$ | Total patches | Patches with channel activated during | | No channel activated |
|---|---|---|---|---|---|
| | | | Intervention | Depolarization | |
| Control | 1 μ*M* | 15 | 0 | 12 | 3 |
| PKC, PMA, ATP | 1 μ*M* | 12 | 1 | 0 | 11 |

(B) *Inactivation of depolarization-activated channels*

| Intervention | $Ca^{2+}$ | Patches with depolarization-activated channels | Patches with channels inactivated |
|---|---|---|---|
| Control | 1 μ*M* | 8 | 0 |
| PKC, DiC8, ATP | <10 n*M* | 5 | 0 |
| PKC, DiC8, ATP | 1 μ*M* | 4 | 4 |
| PKC, PMA, ATP | 1 μ*M* | 15 | 12 |
| PKC, diolein, ATP | 1 μ*M* | 4 | 3 |

**Table 3.** Regulation of $Cl^-$ channels by PKC in CF cells. (**A**) Experiments performed as in Table 1. (**B**) Experiments performed as in Table 2B.

(A) *PKC activation of CF $Cl^-$ channels at low $Ca^{2+}$ concentration*

| Intervention | $Ca^{2+}$ | Total patches | Patches with channel activated during: | | Blank patches |
|---|---|---|---|---|---|
| | | | Intervention | Depolarization | |
| PKC, DiC8, ATP | <10 n*M* | 13 | 0 | 8 | 5 |

(B) *PKC inactivation of CF $Cl^-$ channels at high $Ca^{2+}$ concentration*

| Intervention | $Ca^{2+}$ | Patches with depolarization-activated channels | Patches with channels inactivated |
|---|---|---|---|
| PKC, PMA, ATP | 1 μ*M* | 9 | 6 |

was reversible in three of six patches. Thus PKC-dependent inactivation of $Cl^-$ channels is normal in CF.

Our data allow us to draw several inferences about regulation by PKC of $Cl^-$ channels in normal and CF cells (Fig. 3). The inactivated (*2*) $Cl^-$ channel (Fig. 3A) can be activated by membrane depolarization via an unknown mechanism or by phosphorylation with PKA (Fig. 3B). At low $Ca^{2+}$ concentration, PKC also activates the channel (Fig. 3B). At high $Ca^{2+}$ concentration, PKC maintains the channel in an inactivated state (Fig. 3C): it prevents activation by depolarization; it prevents or attenuates activation by PKC and PKA; and it inactivates channels that were previously activated by depolarization, PKA-dependent phosphorylation, or (low $Ca^{2+}$) PKC-dependent phosphorylation. Both activation and inactivation appear to result from phosphorylation; neither can be explained by down-regulation of PKC.

There are several possibilities to explain the different effects of PKC on the $Cl^-$ channel. The channel may have two different phosphorylation sites for a single PKC, and $Ca^{2+}$ might determine which site is phosphorylated. This could happen in any of several ways. (i) $Ca^{2+}$ might change channel conformation, making different sites accessible to PKC. (ii) Since the $Ca^{2+}$ dependence of PKC can be influenced by the nature of the substrate (*15*), the phosphorylation site on the $Cl^-$ channel might determine the $Ca^{2+}$-dependence of the enzyme. (iii) The interaction of PKC with the membrane might be $Ca^{2+}$ dependent: in the absence of $Ca^{2+}$ PKC could phosphorylate an extrinsic site on the channel (or on a regulatory protein) and in the presence of $Ca^{2+}$ PKC might phosphorylate a site on the channel that is associated with the membrane. Alternatively, different effects could be caused by isozymes of PKC (*16*) that phosphorylate different sites—a $Ca^{2+}$-independent form that activates the channel and a $Ca^{2+}$-dependent form that inactivates the channel. The purified PKC preparations we used probably contained more than one isozyme (*12, 17*). Each of these alternatives require that PKC show substrate specificity (*18*) for two different phosphorylation sites on the channel: one activating and one inactivating. Dual regulatory effects of PKC (stimulation and inhibition) have also been proposed for a cardiac $Ca^{2+}$ channel (*19*).

The demonstration that PKC, in the presence of high $Ca^{2+}$, can cause channel inactivation raises the question of whether an abnormal interaction between PKC and the $Cl^-$ channel is responsible for the regulatory defect in CF. We cannot be certain, but we speculate that this is unlikely because depolarization-dependent activation is normal in cystic fibrosis, but depolarization does not activate channels in the presence of PKC.

The observation that channels from patients with CF cannot be activated by either PKA or PKC at low $Ca^{2+}$ concentration suggests that both enzymes might regulate the $Cl^-$ channel at the same site (*20*). Abnormal regulation by these two enzymes suggests a defect in CF either in the ability of the channel to become phosphorylated or in the mechanism by which phosphorylation results in channel activation.

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18. There are suggestions that PKC isozymes show substrate specificity [K.-P. Huang, F. L. Huang, H. Nakabayashi, Y. Yoshida, *J. Biol. Chem.* **263**, 14839 (1988)]. Substrate specificity for isozymes of catalytic subunit of cAMP-dependent protein kinase has been demonstrated [T. Toda, S. Cameron, P. Sass, M. Zoller, M. Wigler, *Cell* **50**, 277 (1987)].
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20. By "site" we mean a region of the channel or an associated protein. However it is possible that the site might be the same amino acid residue; there is a precedent for phosphorylation of an identical residue by PKC and PKA on tyrosine hydroxylase [K. A. Albert *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7713 (1984); D. G. Campbell, D. G. Hardie, P. R. Vulliet, *J. Biol. Chem.* **261**, 10489 (1986)].
21. We used the excised, inside-out single-channel patch-clamp technique [O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pfleugers Arch.* **391**, 85 (1981)] adapted for airway epithelial cells (*3, 4*). The pipette (external) solution contained 140 m*M* NaCl, 2 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, and 10 m*M* Hepes (*p*H 7.4 with NaOH). The bath (internal) solution contained 140 m*M* NaCl, 2 m*M* $MgCl_2$, and 10 m*M* Hepes (*p*H 7.3 with NaOH). Internal estimated free $Ca^{2+}$ concentration was 1 μ*M* (0.87 m*M* $CaCl_2$ and 1 m*M* EGTA), 150 n*M* (0.5 m*M* $CaCl_2$ and 1 m*M* EGTA), or <10 n*M* (no added $CaCl_2$ and 0.5 m*M* EGTA). Cells were studied at room temperature (21° to 23°C) 1 to 4 days after plating. Voltages are reported in reference to the external surface of the membrane. Cells from canine tracheas, from nasal polyps and tracheas of normal subjects, and from patients with CF were obtained, isolated, and cultured [C. M. Liedtke and B. Tandler, *Am. J. Physiol.* **247**, C441 (1983) and (*4*)]. Cells from eight dogs, five normal humans, and four subjects with CF were used. Patch-clamp studies with dog and normal human cells were identical; therefore they are combined in the tables. $Cl^-$ channels were identified by their *I-V* relation (including conductance and characteristic outward rectification), activation by depolarization, and, in a few cases, by their ion selectivity (*3, 4*).


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