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Research Article

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Activation of Normal and Cystic Fibrosis Cl^- Channels by Voltage, Temperature, and Trypsin

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Abstract

In cystic fibrosis (CF) phosphorylation-dependent activation of outwardly rectifying apical membrane Cl^- channels is defective. To further understand regulation of this channel we examined several other mechanisms of channel activation in normal and CF cells. Previous studies have shown that strong membrane depolarization can activate channels in excised cell-free membrane patches. Here we show that such activation is dependent on both the absolute membrane voltage and the duration of depolarization. Moreover, activation was reversible by membrane hyperpolarization. In some cases, excising patches of membrane from the cell caused channel activation, even in the absence of depolarization. However, the frequency of channel activation with patch excision increased when bath temperature was increased from 23 to 37°C. Although the channel remained in the activated state when temperature was reduced to 23°C, subsequent hyperpolarization inactivated the channel. In cell-attached patches, neither depolarization nor increasing bath temperature to 37°C activated channels, suggesting that neither is physiologically important in regulation of the channel. Thus changes in membrane voltage and bath temperature appear to cause a nonenzymatic change in the channel's conformation; the interactions between voltage and temperature suggest that they may affect the same process. To determine if a proteolytic alteration of the channel could also cause activation, we added trypsin to the cytosolic surface of excised membrane patches. Trypsin activated channels, which could not then be inactivated by either hyperpolarization or phosphorylation with PKC, suggesting that trypsin removed or altered a region of the channel involved in inactivation. All of these interventions activated Cl^- channels from both normal and CF cells. Thus many aspects of Cl^- channel activation are normal in CF; only phosphorylation-dependent activation is defective.

Introduction

Apical membrane Cl^- channels control, in part, the rate of Cl^- secretion in airway epithelia (1). In cystic fibrosis (CF)[1] airway epithelia regulation of these outwardly rectifying Cl^- channels is defective, resulting in a Cl^- impermeable epithelium. Previous studies have focused on regulation of Cl^- channels by three second messenger systems: (*a*) phosphorylation by cAMP-dependent protein kinase (PKA) causes activation of normal but not CF Cl^- channels (2, 3); (*b*) phosphorylation by protein kinase C (PKC) has dual effects that depend on the experimental conditions; at low $[Ca^{2+}]$ PKC activates normal but not CF channels, whereas at 1 μM $[Ca^{2+}]$ PKC inactivates both normal and CF Cl^- channels (4, 5); and (*c*) an increase in $[Ca^{2+}]_c$ produced by Ca^{2+} ionophores can activate both normal and CF Cl^- channels, although the channel is not directly gated by Ca^{2+} in either excised or cell-attached patches (6–8).

In addition to these possibly physiologic means of activating the channel, we and others (2, 3) have reported that in excised, inside-out patches, normal and CF Cl^- channels can be activated by strong membrane depolarization. The purpose of this work was to further investigate this and other nonphysiologic mechanisms of channel activation[2] because they may give us insights into the structural properties of the channel. In addition, a comparison of normal and CF Cl^- channels may provide further information about the channel's function in this genetic disease.

Methods

Cells were isolated from dogs and CF and non-CF human airway epithelia (trachea and nasal polyps) and cultured on collagen-coated plastic coverslips as we have previously described (9, 10). For experiments on the effect of voltage and trypsin, studies of normal human and CF cells are shown; for experiments on the effect of temperature, studies of canine and CF cells are shown; for all three types of studies, similar results have been obtained with dog and normal human cells. Experiments were performed at room temperature and ambient CO_2 2–5 d after seeding. For some studies temperature in the bath was measured with a thermistor and controlled at either 23 or 37°C.

For excised, inside-out patch-clamp studies we used the same methods we have previously described (10). Cl^- channels were identified by their size and characteristic outward rectification. The external (pipette) solution contained (in mM) 140 NaCl, 2 $MgCl_2$, 2 $CaCl_2$, and 10 Hepes (pH 7.4 with NaOH). The internal (bath) solution was the same except that it contained 1 mM EGTA and 62 μM $CaCl_2$ (estimated free $[Ca^{2+}]$, 10 nM). For studies using trypsin the $[Ca^{2+}]$ was 1 mM, although similar results were obtained with 10 nM Ca^{2+}. Voltages are referenced to the external surface of the membrane; thus, a positive voltage is depolarizing. We frequently applied voltage steps, or pulses; we refer to the voltage between steps as the "holding voltage."

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1. *Abbreviations used in this paper:* CF, cystic fibrosis; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

2. The Cl^- channel functions in at least two modes. We refer to an inactivated channel as one that is unstimulated or quiescent and always in the closed state; i.e., the probability of being in the open state, P_o, = 0. An activated channel is one that has been stimulated and spontaneously flickers back and forth between the open and closed states; i.e., $P_o > 0$.

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Results and Discussion

Activation by depolarization. It has previously been shown that large depolarizing voltages can activate Cl^- channels in excised, inside-out patches (2, 3). To further investigate this phenomenon we examined the relationship between absolute voltage and the duration of depolarization. In excised patches prolonged depolarization to high voltages caused loss of the pipette-membrane seal; therefore, membrane voltage was held at −40 mV and intermittently stepped to depolarizing voltages (step duration, 2 s; interval, 2 s). The first series of depolarizing steps was to 40 mV; if no channel was activated, the depolarizing voltage was increased to 80 mV; if still no channel was activated, we progressed to 120 and then to 160 mV. In sequential patches we varied the time at each voltage by varying the number of pulses using 4, 25, or 50 pulses (i.e., 8, 50, or 100 s).

Fig. 1 shows that activation of Cl^- channels was a function of both absolute membrane voltage and the duration of depolarization. When the patch was depolarized to each voltage for 8 s most channels were activated at 160 mV. In contrast, when the patch was depolarized to each voltage for 100 s most channels were activated at 80 mV. At an intermediate time intermediate voltages were required. Fig. 1 also shows that in a few cases channels spontaneously activated with excision and were already in the activated state during the first step to 40 mV (two patches in the 8-s group and two patches in the 100-s group). This spontaneous activation after excision occurred even though membrane voltage was −40 mV. The percent of channels spontaneously activating after excision varied from day to day and from culture to culture. The observation that depolarization was not always required for activation points out the necessity of using paired controls when investigating the effect of an experimental manipulation on channel activation.

We also asked if membrane depolarization would activate channels in cell-attached patches. Because cell-attached patches are exposed to the membrane voltage of the cell, we clamped voltage at 120 mV, which corresponded to a voltage across the membrane patch of ~ 60 mV.[3] The results are shown in Fig. 2. Patches were studied during three sequential periods. In one group the patch was excised and membrane voltage was continuously held at 60 mV for 4 min; if no channel activated, membrane voltage was held at 60 mV for a second 4-min period; if still no channel activated in the patch, membrane voltage was progressively depolarized to higher voltages until a channel activated. The results show that most channels in excised patches activated during the first period (60 mV). In the other group, cell-attached patches were continuously depolarized to 120 mV during the first 4-min period; if no channel activated, the patch was excised and continuously held at 60 mV for 4 min; if still no channel activated in the patch, membrane voltage was progressively depolarized. The results show that depolarization did not activate channels in cell-attached patches even though channels were present in the patch, and even though a similar voltage was sufficient to activate channels in excised patches. We have also depolarized the membrane of cell-attached patches to higher voltages without activating channels (data not shown). Thus, activation of Cl^- channels by depolarization is only observed in the excised patch.

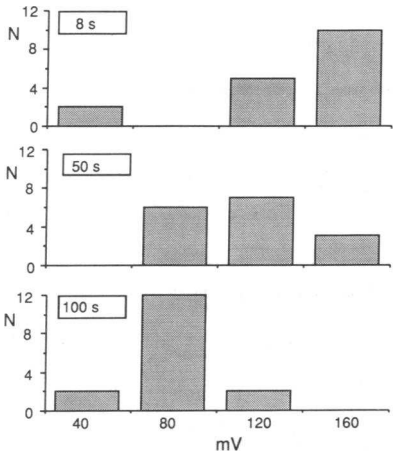

Figure 1. Effect of membrane voltage and time on Cl^- channel activation. Excised patches were held at −40 mV and depolarized to the various voltages for the indicated total durations of time, as described in text. Data show the number of patches (*N*) in which a Cl^- channel activated at the voltage shown on the *x* axis. Data from patches that contained no channels are excluded.

As previously reported (2, 3), once a channel was activated by depolarization, it was maintained in an activated state even at less depolarizing voltages. To determine if depolarization-induced activation was reversible, we progressively hyperpolarized the membrane. In some cases strong membrane hyperpolarization caused a loss of the pipette-membrane seal before channels inactivated. However, in most cases we were able to inactivate channels by hyperpolarizing the membrane; just as with depolarization-dependent activation, the effect was time dependent. We show a representative example in Fig. 3 where we plot membrane voltage and indicate the state of the channel (activated or inactivated) during sequential 2-s sweeps. (By "activated" we mean that the channel was observed to be in the open state during some portion of the 2-s period, i.e., $P_o > 0$). The patch was excised and a Cl^- channel was activated after multiple depolarizing steps to 120 mV. Then we applied

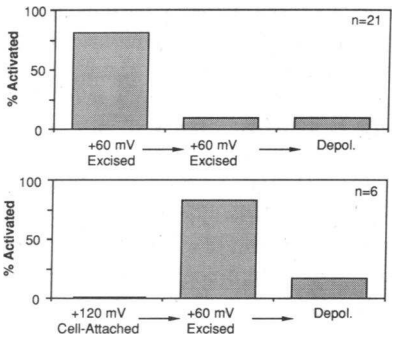

Figure 2. Effect of membrane voltage on Cl^- channel activation in cell-attached and excised patches. Two groups of patches were studied. For those shown in the top panel, the patch was excised and voltage was continuously held at 60 mV for two consecutive 4-min periods. If no channel activated during this time the membrane was progressively depolarized (*Depol.*) to activate any other channels in the patch. For those shown in the bottom panel, membrane voltage was held at 120 mV in cell-attached patches for 4 min. After 4 min the patch was excised and voltage was continuously held at 60 mV for 4 min. If no channel activated during this time the membrane was progressively depolarized. Data are percent of total channel containing patches with a channel activated at each condition. The number of channels activated during the first period is significantly different for the two groups ($P < 0.002$ by chi square analysis).

3. We used −60 mV as an estimate of membrane voltage; membrane voltage measured with conventional microelectrodes ranges from −45 to −60 (1). We also chose continuous depolarizing voltages of 60 mV excised and 120 mV cell attached because at these voltages it was possible to complete the experiment without losing the pipette-membrane seal.

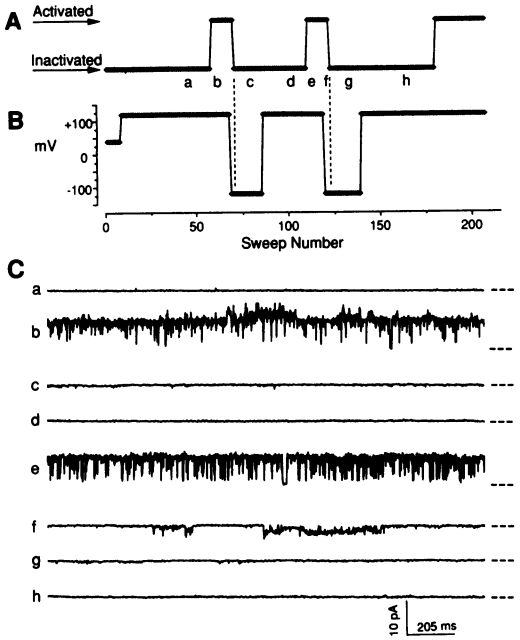

Figure 3. Reversibility of voltage-induced Cl^- channel activation. *A* shows state of the channel (activated or inactivated).[2] *B* shows membrane voltage. *C* shows current tracings recorded at the points (*a–h*) indicated in *A*. Data points are for sweeps of 2-s duration (2-s interval between sweeps). For depolarizing voltage steps holding voltage was −40 mV and for hyperpolarizing voltage steps holding voltage was −80 mV. Dashed line between *A* and *B* shows the time of transition from the activated to the inactivated state. In *C*, the dashed line shows zero current level. Sweeps, *a*, *b*, *d*, *e*, and *h* were obtained at 120 mV; sweeps *c*, *f*, and *g* were obtained at −120 mV.

hyperpolarizing steps to −120 mV. After a few steps to −120 mV the channel inactivated and remained in an inactivated state even when membrane voltage was no longer hyperpolarizing. Reactivation of the channel once again required a series of steps to 120 mV. After the channel had activated the sequence was repeated again, showing complete reversibility.[4] The absolute voltage (−60 to 200 mV) and time (a few seconds to minutes) required to inactivate Cl^- channels varied between preparations. We also found that hyperpolarization could inactivate channels that activated spontaneously with excision. Fig. 4 shows results from a series of similar experiments. When membrane voltage was maintained at −40 mV channels did not inactivate, consistent with our previous experience (5), but when voltage was more hyperpolarized than −80 mV, most channels inactivated.

Several observations indicated that depolarization-induced activation was not a physiologic process. First, it was not observed in cell-attached patches. Second, secretagogues produced only relatively small changes in apical membrane voltage (1), but more importantly, the change in voltage was

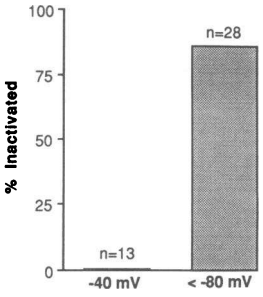

Figure 4. Inactivation of Cl^- channels by hyperpolarization. In one group of patches membrane voltage was maintained by −40 mV and in the other voltage was stepped (holding voltage −40 or −80 mV) to values more negative than −80 mV. Data are percent of activated channels that inactivated during a 5-min study period.

caused by activation of Cl^- channels, not the converse. Third, in native airway epithelia changes in apical voltage caused by imposition of transepithelial currents did not affect apical conductance (11).

We do not know the mechanism by which large voltages activate and inactivate Cl^- channels. The conditions we used and our finding that activation by depolarization is reversible suggested that this process is unlikely to be mediated by protein phosphorylation or dephosphorylation, or by an interaction of $[Ca^{2+}]$ with the channel. Because some channels activate spontaneously with excision, and because depolarization-induced activation was only observed in excised patches, we considered the possibility that activation resulted from dissociation of some regulatory or inhibitory factor. However, the reversibility of the process makes this suggestion less likely. We speculate that depolarization and hyperpolarization in an excised patch may cause a physical change in channel conformation. There may be an energy barrier separating two relatively stable states (inactivated and activated). Depolarization and hyperpolarization may provide the energy required to move the channel back and forth over the energy barrier between these two states.

This notion seems reasonable because the channel is a charged protein that spans the lipid bilayer and could therefore be influenced by the large electric field strength across the membrane (when a patch is depolarized to 120 mV the electric field strength is $\sim 9 \times 10^7$ V/m away from the resting value). However, the failure of voltage to activate cell-attached channels indicates that voltage alone is insufficient. In any case, activation and inactivation by large membrane voltages appears to be a relatively unique feature of these outwardly rectifying Cl^- channels.

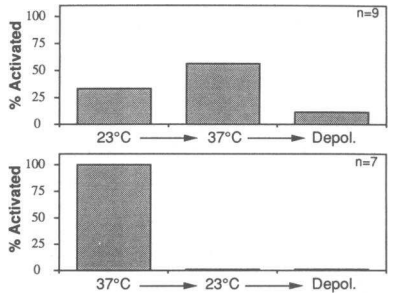

Figure 5. Effect of temperature on Cl^- channel activation. Membrane voltage was held at −40 mV and intermittently stepped to 40 mV (duration, 2 s; interval, 2 s). Patches were excised into a bath at either 23 or 37°C. After 2 min temperature was switched to the other value and remained there for 2 min after temperature had reached the target value. The time to complete the change in temperature (measured with a thermistor in the bath) was ~ 1 min. If no channel activated in the patch the membrane was progressively depolarized (*Depol.*). Data are percent of total channel-containing patches that contained a channel activated at each condition. Number of blank patches was similar for the two protocols: 5 and 6, respectively.

4. Note that we do not discuss the effect of voltage on P_o. In unpublished studies we found that in the range of ±80 mV there was little consistent effect of voltage on P_o. In some cases P_o decreased with depolarization, in some cases P_o increased with depolarization, but in most cases there was little effect of voltage on P_o. At more depolarized or hyperpolarized values we found that voltage could more frequently alter P_o, although again in some cases P_o decreased with hyperpolarization (e.g., tracing *f* in Fig. 3), and in some cases P_o decreased with depolarization (17).

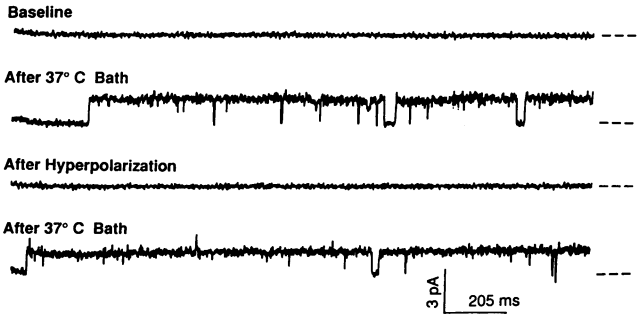

Figure 6. Example of the interaction between temperature and voltage on Cl^- channel activation. All tracings were obtained at 40 mV and 23°C. Representative recordings from a human airway cell are shown under baseline conditions, after raising bath temperature to 37°C for 150 s and then returning to 23°C, after hyperpolarization to −120 mV for 12 s, and after bath temperature was increased to 37°C for 180 s and then returned to 23°C.

Activation with temperature. As indicated above, the Cl channel sometimes activated spontaneously after excision (even at hyperpolarizing voltages), but in most cases depolarization was required for activation. In another report, Cl channels from several cell types activated after excision without requiring depolarization (12). One difference between those experiments and ours is that patches were studied at 37°C, whereas we studied patches at 23°C.

To examine the effect of temperature on channel activation we studied alternate patches with the following protocol. One group was excised into a bath solution at 23°C. Fig. 5 shows that these patches a channel activated during the first 2-min period in one-third of the patches that contained channels. If no channel activated, bath temperature was increased to 37°C. The increased temperature activated channels in all of the remaining patches, except for one patch that required depolarization to activate the channel. The other group of patches was excised into a 37°C bath. Channels were activated in every patch that proved to contain a Cl^- channel as evidenced by a lack of further channel activation with depolarization. The effect of a 37°C bath on channel activation was confined to the excised patch because patches studied in the cell-attached mode at 37°C did not show channel activation. Once channels were activated by exposure to a 37°C bath they remained in the activated state even when the bath temperature was reduced to 23°C.

If membrane voltage and an increased temperature were affecting a similar activation/inactivation process that controlled the channel, then the activated state produced at 37°C might be reversible. To test this possibility we asked whether channels that had been activated with temperature could be inactivated by hyperpolarization. Fig. 6 shows a tracing of a channel activated by a 37°C bath. The bath temperature was reduced to 23°C and then the membrane was hyperpolarized to −120 mV. After 4 s of hyperpolarizing sweeps the channel inactivated. When the bath temperature was once again increased to 37°C the channel activated. At 37°C the channel could not be inactivated by hyperpolarization (not shown). Similar results were obtained in five other patches.

These results suggest that exposing a patch to a 37°C bath causes a nonenzymatic change in channel conformation. However, work with renal membrane vesicles suggested that an increase in temperature might activate a phosphatase that could activate a Cl^- conductance (13). To test the possibility that the effect of temperature resulted from phosphatase activity we examined the effect of a 37°C bath in the presence of phosphatase inhibitors. For these studies the internal solution was altered by replacing 50 mM NaCl with 50 mM NaF and adding 0.1 mM Na vanadate and 1 mM EDTA (no Ca^{2+} was added to the solution). In two patches studied in the presence of these inhibitors, increasing the bath temperature to 37°C caused channel activation that could be reversed by returning to 23°C and hyperpolarizing the patch. When bath temperature was again increased to 37°C the channels reactivated.

We also examined the effect of temperature on CF Cl^- channels. Patches were excised at 23°C and if no channel was observed to be open in the patch the temperature was increased to 37°C. If no channel activated within 2 min the patch was progressively depolarized. Alternate patches were studied under control conditions (23°C) using the same time intervals. Fig. 7 shows that exposure to a 37°C bath activated excised CF Cl^- channels. (Note that Fig. 7 also shows the effect of trypsin, which is discussed below.) As previously observed with studies of transepithelial Cl^- secretion in the native epithelium and cultured monolayers (7, 8), we found that increasing temperature to 37°C did not activate Cl^- channels in cell-attached patches.

Activation of channels by an increase in bath temperature to 37°C was not a physiologic response, because it occurred only in excised patches. We do not know how increasing the temperature to 37°C causes channel activation. We previously speculated that the channel might be tonically inhibited and that excising a patch might remove the inhibition (14). At first inspection it seemed that increasing temperature in an excised patch might cause a change in an inhibitory component of the

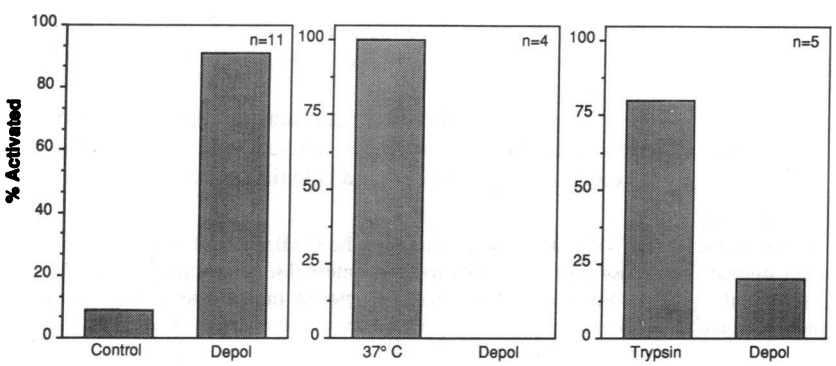

Figure 7. Effect of temperature and trypsin on activation of CF Cl^- channels. Patches were excised at 23°C and if no channels were observed to open in the patch, they were studied under three conditions in an alternate fashion. During the first 2-min period they were exposed to a 37°C bath (*37°C*), 0.05% trypsin (*Trypsin*), or no intervention (*Control*). If no channel activated during the 2-min period, the membrane was progressively depolarized (*Depol.*) to determine if a channel was present. Data are the percent of patches containing a channel that activated under each condition; *n* is the number of patches that contained Cl^- channels. The number of blank patches was similar for all three conditions: 2, 0, and 1, respectively.

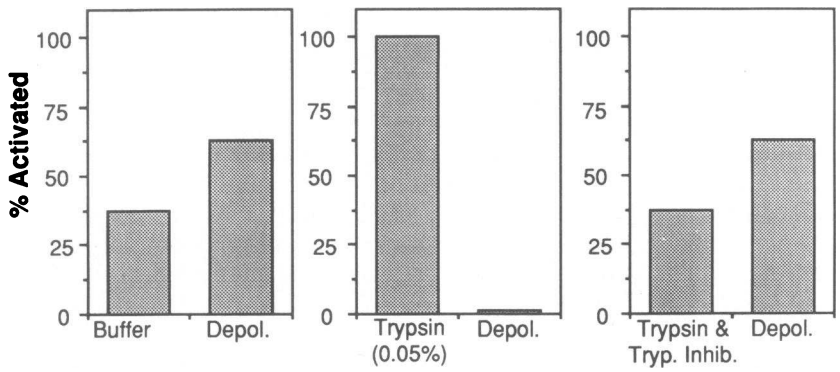

Figure 8. Effect of trypsin on Cl^- channel activation. Holding voltage was −40 mV and voltage was intermittently stepped to 40 mV (duration, 2 s; interval, 2 s). There were two study periods. During the first 4-min period buffer (*Control*), trypsin (0.05%), or trypsin plus trypsin inhibitor (0.025%) were added to the internal surface of the patch. During the second period the membrane was progressively depolarized (*Depol.*) to activate any channels in the patch. Data are percent of total patches with a channel activated at each condition; number of patches containing a Cl^- channel was 8 for each group; number of blank patches was similar for each group: 7, 10, and 7, respectively.

channel or activate a membrane-associated phosphatase or protease that could activate the channel. However, the lack of effect of a phosphatase inhibitor, the reversibility, and the interaction with membrane voltage suggest that this is not the case. Rather, the results suggest that an increased temperature causes a nonenzymatic change in channel conformation. It is interesting to speculate whether voltage and temperature affect a similar process that regulates the channel.

Activation by trypsin. We also investigated the effect of trypsin on the channel. Our rationale was based on several observations: (*a*) PKA activates Cl^- channels by phosphorylation, and the phosphorylation sequence for PKA is Arg-Arg-X-Ser-; (*b*) trypsin specifically cleaves proteins at the basic residues lysine and arginine; and (*c*) there are numerous precedents for trypsin-induced activation of enzyme systems when trypsin cuts at the phosphorylation site, and trypsin has been shown to activate (or remove inactivation of) other channels that are regulated by phosphorylation (15, 16).

Fig. 8 shows that in control patches exposed to buffer alone channels activated spontaneously in some patches. However, addition of 0.05% trypsin to the internal surface of the patch caused channel activation in all patches that contained a Cl^- channel; if no channel was activated by trypsin, none was activated on subsequent depolarization. Patches exposed to trypsin plus trypsin inhibitor (0.025%) had a similar frequency of channel activation as controls, indicating that trypsin activated channels by proteolysis.

The current-voltage relationship and the single-channel conductance of trypsin-activated channels were the same as for channels activated by other maneuvers. Channels activated by trypsin also remained anion selective (n = 4): changes in the internal Cl^- concentration caused a shift in reversal potential, indicating that the channel was nine times more selective for Cl^- than for Na^+, a value similar to that previously reported (14).

We had previously shown that phosphorylation of the Cl^- channel by PKC at a high $[Ca^{2+}]$ (1 μM) caused inactivation of channels that had previously been activated by depolarization, phosphorylation by PKA, or phosphorylation by PKC at a low $[Ca^{2+}]$ (< 10 nM) (5). To determine if PKC inactivates trypsin-activated channels, we activated three channels with trypsin then washed away the trypsin and added purified PKC, ATP (1 μM), and dioctanoylglycerol (1 mg/ml) to the internal surface of the patch at 1 mM $[Ca^{2+}]$ as previously described (5). However, in none of the three did the phosphorylation solution inactive the channel. In one other patch a depolarization activated channel was inactivated by addition of PKC plus diolein (40 μg/ml) and ATP and was then activated by addition of trypsin.

Because hyperpolarization inactivated channels that had been activated by depolarization or a 37°C bath, we examined its effect on trypsin-activated channels. Despite prolonged hyperpolarization this maneuver failed to inactivate channels in five of six patches that had been exposed to trypsin.

We also asked whether trypsin would activate CF Cl^- channels; Fig. 7 shows that it did.

It is possible that trypsin activates Cl^- channels by cleavage at a phosphorylation site but many other sites of cleavage are also possible because this ion channel probably has basic residues at the channel mouth.[5] However the lack of an effect of trypsin on channel conductive properties suggests that the cleavage site is in a regulatory domain involved in channel inactivation.

Summary. In Table I we summarize the various interventions that cause Cl^- channel[6] activation or inactivation. The present and previous data indicate that the conductive portion

Table I. Summary of Interventions That Cause Cl^- Channel Activation or Inactivation

Intervention	Condition	Non-CF	CF
PKA	Attached, excised	Activation	No effect
PKC, low $[Ca^{2+}]$	Attached, excised	Activation	No effect
PKC, high $[Ca^{2+}]$	Attached, excised	Inactivation	Inactivation
A23187 and Ca^{2+}	Attached	Activation	Activation
Depolarization	Excised	Activation	Activation
Hyperpolarization	Excised	Inactivation	Inactivation
Patch excision (23°C)	Excised	Activation	Activation
Temperature (37°C)	Excised	Activation	Activation
Trypsin	Excised	Activation	Activation

Attached, Channels studied with any of the following: transepithelial measurements (short-circuit current or $^{36}Cl^-$ flux), $^{125}I^-$ efflux, or the cell-attached patch-clamp technique. *Excised,* Cl^- channels studied in excised, inside-out patches. As indicated in the text, excision at 23°C only occasionally causes channel activation, but the results are similar in normal and CF cells.

5. Two other anion channels, the GABA and glycine receptors have basic residues at the presumed internal surface of the channel (18, 19).
6. For the sake of clarity we refer to the channel as a single entity. However, it is possible that it may contain more than one subunit or associated regulatory protein.

of the Cl^- channel protein is normal in CF. Activation caused by phosphorylation with PKA and PKC at low $[Ca^{2+}]$ are the only two interventions known to be defective in CF (2–5). Another phosphorylation-dependent effect, inactivation by PKC at high $[Ca^{2+}]$, is normal in CF (5). Activation with another intervention that could potentially be physiologically important in regulating the channel, an increase in $[Ca^{2+}]_c$, is intact in CF (7, 8). In addition, activation by three interventions that are probably not of physiologic importance and are only observed in excised patches (depolarization, 37°C temperature, and trypsin) are normal in CF cells.

Thus, in CF cells the conductive properties of the Cl^- channel and all aspects of channel activation appear to be normal except activation by phosphorylation. These observations suggest that only a single mechanism of channel activation is defective in CF and that there is a CF abnormality either in the phosphorylation site itself or in a step distal to phosphorylation. If, as we speculated above, trypsin were activating the channel by proteolysis at or near the phosphorylation site, it would suggest that in CF the mechanisms lying between the phosphorylation site and the change in protein conformation that causes channel activation are intact.

Acknowledgments

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