antibody or pharmacologic agents. If feasible, such therapy might be more specific and less toxic than currently available modalities.

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21. For example, mouse gut intraepithelial lymphocytes, a mucosal effector cell population, bind exclusively to mucosal (Peyer's patch) HEV and not to lymph node HEV, thus explaining their specific localization to mucosal surfaces (M. Schmitz, D. Nunez, E. C. Butcher, in preparation). Human lamina propria blast cells exhibit a similar specificity for mucosal HEV (S. Jalkanen, R. MacDermott, J. Nash, E. C. Butcher, unpublished results).
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Altered Regulation of Airway Epithelial Cell Chloride Channels in Cystic Fibrosis

RAYMOND A. FRIZZELL,* GERHARD RECHKEMMER, RICHARD L. SHOEMAKER

In many epithelial cells the chloride conductance of the apical membrane increases during the stimulation of electrolyte secretion. Single-channel recordings from human airway epithelial cells showed that β-adrenergic stimulation evoked apical membrane chloride channel activity, but this response was absent in cells from patients with cystic fibrosis (CF). However, when membrane patches were excised from CF cells into media containing sufficient free calcium (approximately 180 nanomolar), chloride channels were activated. The chloride channels of CF cells were similar to those of normal cells as judged by their current-voltage relations, ion selectivity, and kinetic behavior. These findings demonstrate the presence of chloride channels in the apical membranes of CF airway cells. Their regulation by calcium appears to be intact, but cyclic adenosine monophosphate (cAMP)–dependent control of their activity is defective.

EVIDENCE OF A DEFECT IN CHLORIDE transport across epithelial cells in patients with cystic fibrosis (CF) was published by Quinton (*1*) who used ion-replacement studies to show that the elevated transepithelial voltage across isolated, perfused CF sweat ducts was due to an abnormally low Cl^- permeability. Knowles *et al.* (*2*) found that a Cl^- diffusion potential could be generated across normal, but not CF, airway mucosa during superfusion with solutions of low Cl^- concentration. These findings were confirmed by Widdicombe, Welsh, and Finkbeiner (*3*) for primary cultures of tracheal cells. Monolayers from CF patients failed to show an increase in the current associated with Cl^- secretion when they were exposed to isoproterenol or prostaglandin E_2. Unlike the situation in normal cells, the apical membrane potential of CF cells was not depolarized by secretory stimuli that evoke an increase in apical membrane Cl^- conductance. Thus, impaired apical Cl^- conductance may limit water and electrolyte secretion and thereby contribute to the accumulation of mucus that compromises the pulmonary function of CF patients. Nevertheless, it was not apparent whether the reduced expression of airway cell Cl^- conductance resulted from an absence or impairment of the conductance mechanism per se, or from a defect in its regulation.

We applied patch-clamp techniques (*4*) for single-channel recording to upper airway cells in primary culture to determine whether the decreased Cl^- conductance of CF cells was due to alterations in the presence, properties, or regulation of single Cl^- channel activity. This approach required relatively little material, in contrast to isotopic flux determinations or transepithelial measurements. We used primary cell culture because it is unlikely that the secondary effects of inflammatory or immune responses would be expressed in culture. The culture methods (*5*) were similar to those described by Widdicombe and co-workers (*6*). We noted, as have others (*3*), no readily apparent differences in the morphologic appearance or growth characteristics of cells derived from CF airways.

During cell-attached recording from normal cells, the β-adrenergic stimulant epinephrine evoked Cl^- channel activity after a delay that was partly due to diffusion of this β-agonist from the site of addition to the recording site (Fig. 1). The average delay between agonist addition and initiation of channel activity was approximately 3 minutes. All of the membranes that we studied were electrically quiet before addition of agonist, so that epinephrine induced single-channel activity in a previously inactive membrane. Unitary events of two different conductance levels could be discerned during cell-attached stimulation by epinephrine (Fig. 1). Both conductance levels were observed frequently in the same membrane patch but were also present individually in separate recordings. Responses identical to that shown in Fig. 1 were obtained upon addition of an analog of cyclic

Department of Physiology and Biophysics and Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham, Birmingham, AL 35294.

*To whom correspondence should be addressed.

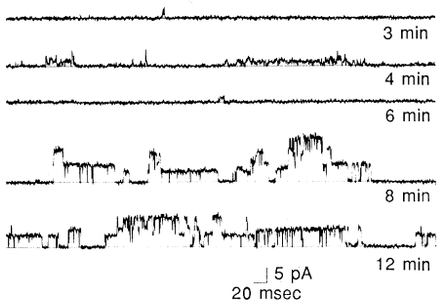

Fig. 1. Time course of activation of chloride channels in a normal airway cell by epinephrine. The results are typical of 16 experiments on cells from 8 normal patients. Each cell-attached recording is approximately 600 msec in length; successive records were obtained at the times shown at the right margin after epinephrine (5 μM) was added to the bath. The records were obtained at a holding potential of +80 mV; the polarity of transmembrane voltages is expressed with reference to the pipette solution. A similar convention applies to statements on membrane sidedness; "inside" is the cytoplasmic side.

adenosine monophosphate (cAMP) to the bath (8-bromo–cAMP, 0.1 mM). No differences in the onset of activity and effectiveness of epinephrine or cAMP were noted. When the results obtained with either stimulus were pooled, cell-attached activation occurred in 54 percent of the membrane patches (activation by epinephrine or cAMP was obtained in 15 of 28 experiments with cells from eight normal individuals). Chloride channel activity could be evoked in single cells, from cells growing at the edges of cell groups, or from cells in confluent sheets where only the apical membrane was accessible to the recording pipette.

In contrast to these findings on normal cells, no Cl^- channel activity was elicited by epinephrine, cAMP, or forskolin (0.1 mM) during cell-attached recording from cells derived from the airways of CF patients. In normal cells, the longest time required for activation was 8 minutes. We routinely waited at least 10 minutes for each activator to elicit a response in CF cells; on many occasions these substances were added sequentially, with 10-minute periods between each addition. Epinephrine or cAMP failed to evoke Cl^- channel activity in 32 experiments on cells from four CF patients. Since 54 percent of normal cells were activated under these conditions, about 17 of these attempts should have succeeded if the CF cells were responding normally.

However, chloride channel activity could be elicited when membrane patches from CF cells were excised into Hepes-buffered Ringer solution. The time required for activation ranged from immediate to several minutes after excision, that is, after the formation of an inside-out patch. The longest period required for activation was 6 minutes. Channel activation as a result of excision of the membrane patch was also obtained in control and CF cells that had not been exposed previously to epinephrine or cAMP.

We compared the current-voltage (I-V) characteristics of channels in membrane patches excised from CF and control cells (Fig. 2). Both high and low conductance levels were observed in CF cells, and their I-V relations were identical to those obtained from control cells. Both channels were anion selective. As judged from I-V relations obtained with imposed ion gradients, they were at least ten times more selective for Cl^- than Na^+ (*7*). The high conductance channel (Fig. 2A) had a nonlinear I-V relation; the slope conductance was greater at depolarizing voltages and, at the reversal potential of 0 mV, was approximately 50 pS. Its kinetics were complex, showing rapid transitions (flickering) between open and closed states during predominantly open periods (see record illustrated in Fig. 5). These transitions are not apparent in Figs. 1, 3, and 4 because they were rapid and the data were filtered at 3 kHz for illustration. The low conductance channel (Fig. 2B) had a linear I-V relation with a single-channel conductance of approximately 20 pS and straightforward, open-closed kinetic behavior. Under these conditions, the kinetic behavior of control and CF Cl^- channels did not differ.

We next tested whether activation of Cl^- channels in excised CF patches was due to the high concentration of calcium in the bath to which the inside of the membrane was exposed on excision. Chloride channel activity, which had been stimulated by excision of membrane patches from CF cells into solutions containing 500 nM free Ca^{2+}, was inhibited by addition of EGTA to the bath (Fig. 3). Three to four minutes were required for complete cessation of activity of the three 50-pS channels that appeared after excision. At least part of this delay was due to diffusion of EGTA to the pipette tip holding the inside-out patch; this may not be the only explanation because the time course of activation upon excision into a bath containing high concentrations of Ca^{2+} was variable (see above).

An analogous experiment was performed by excising membrane patches into solutions containing various Ca^{2+}-EGTA buffers. Excision of a CF patch into a solution of approximately 20 nM free Ca^{2+} did not evoke channel activity (Fig. 4). At this Ca^{2+} concentration, the recording was continued for 10 minutes to ensure that activation was not delayed. Subsequently, the free Ca^{2+} concentration was raised to approximately 180 nM, and activity of the 20-pS Cl^- channel was evident. After 1 minute, activity of the 50-pS channel was also apparent. When the free Ca^{2+} was increased to approximately 760 nM, increased activity of both channel types was observed.

The Cl^- channels of CF cells could also be activated during cell-attached recording by addition of the Ca^{2+} ionophore A23187 (Fig. 5). The cells were bathed by Ringer solution containing 1 mM Ca^{2+}, and the ionophore elicited activation with a delay that was similar to that observed with epinephrine or cAMP. As in the inside-out patch, activity of both the 20- and 50-pS channels could be evoked by A23187 during cell-attached recording (Fig. 5). Results similar to those illustrated in Figs. 3 to 5, obtained with CF cells, were also obtained from normal cells. In the absence of epinephrine or cAMP, Cl^- channels of normal cells could be activated during cell-attached recording by A23187 or by exposure of inside-out membrane patches to free Ca^{2+} concentrations of 180 nM or greater. There were no obvious differences in the Ca^{2+}-dependent activation of normal and CF cell Cl^- channels.

Our findings illustrate the presence of Cl^- channels in CF cells and demonstrate that their biophysical properties are indistinguishable from those of normal cells. A

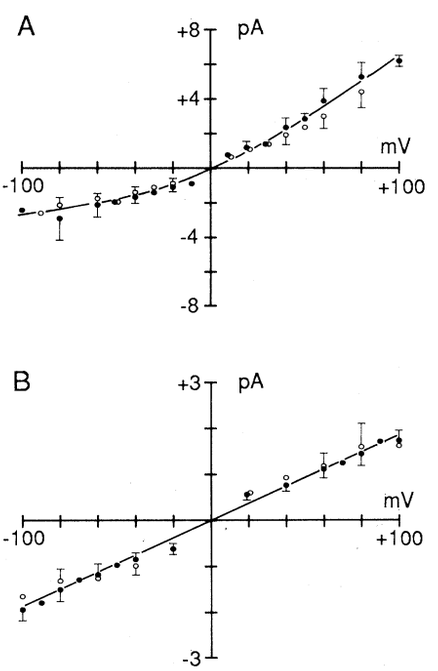

Fig. 2. Current-voltage relations of the (A) high and (B) low conductance channels recorded in excised, inside-out membrane patches. The bath contained either modified Ringer solution or 150 mM NaCl solution with a Ca^{2+}-EGTA buffer (free Ca^{2+}, 500 nM); their compositions are in (*4*). Open circles, normal cells; closed circles, CF cells. Number of experiments for (A): 6, control; 9, CF; (B): 4, control; 12, CF. The lines describing the data were drawn by eye.

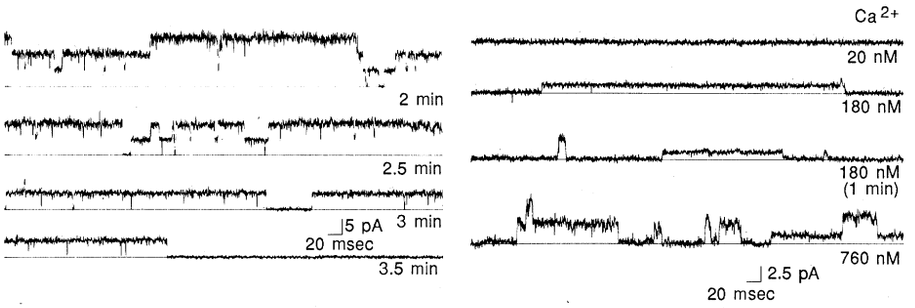

Fig. 3 (left). Time course of inhibition by EGTA of chloride channel activity in an inside-out patch from a CF cell. These results are typical of data from four experiments on cells from two CF patients and from three experiments on cells from two control patients. This patch was excised into 150 m*M* NaCl containing 500 n*M* free Ca^{2+}, and channel activity began approximately 3 minutes after excision. This solution was partially replaced by a 20 n*M* Ca^{2+}-EGTA–buffered solution to lower the free Ca^{2+} to approximately 200 n*M*. Channel activity continued under these conditions. At the beginning of the experiment (0 min), 1 m*M* EGTA was added to the bath to lower the free Ca^{2+} to approximately 20 n*M*. After 3.5 minutes no further channel activity was recorded. The holding potential was +60 mV throughout. Fig. 4 (right). Activation of chloride channel activity by Ca^{2+} in an inside-out patch from a CF cell. The results are typical of data from 25 experiments on cells from three CF patients and from nine experiments on cells from four control patients. The membrane was excised into 150 m*M* NaCl containing a 20 n*M* Ca^{2+}-EGTA buffer, which was subsequently replaced by similar solutions that were buffered to 180 and 760 n*M* Ca^{2+} as indicated. The holding potential was +60 mV throughout.

pathway for Ca^{2+}-induced Cl^- channel activation is functional in both normal and CF cells. Activation could be elicited by addition of Ca^{2+} ionophore during cell-attached recording, or by excision of membrane patches into Ca^{2+}-containing media. The mechanistic basis of Ca^{2+}-induced activation requires further evaluation but does not appear to involve phosphorylation, since it occurs with excision or when Ca^{2+} is added to inside-out membrane patches bathed by media containing no added adenosine 5′-triphosphate (ATP). This mode of activation might be mediated by calmodulin or a Ca^{2+}-dependent dephosphorylation process.

Our findings also demonstrate that the pathway of cAMP-induced activation of Cl^- channel activity, normally elicited by β-adrenergic activation of airway epithelial cells, is defective. In no instance was it possible to stimulate channel activity in CF cells with epinephrine or cAMP. These findings suggest that the defect in CF airway cells does not lie in the Cl^- conductance pathway per se, but rather in the regulation of this pathway by a cAMP-dependent process. Evidence for an alteration in the regulatory pathway emerged also from the data of Sato and Sato (*8*) who compared the cholinergic and β-adrenergic control of sweat secretion from secretory coils isolated from control and CF subjects. They found that a normal level of sweat secretion was stimulated by cholinergic agonists, which have effects mediated by increased intracellular Ca^{2+} concentrations (*9*). In contrast, stimulation by isoproterenol was absent in CF secretory coils, even though normal increases in intracellular cAMP concentrations were observed. The latter finding has been confirmed for cultured airway cells (*10*, *11*). A defect in the regulatory pathway may explain the selective alterations that occur in specific secretory organs in CF—the airways, pancreas, and sweat glands. A regulatory defect may also account for the diversity of changes in cellular events, which range from decreased anion conductance to altered chemistry of secreted mucins (*12*).

Our findings suggest that further attempts to localize the defect in CF should focus the steps between β-agonist–induced increases in intracellular cAMP concentrations and activation of Cl^- channel activity. The normal cAMP-dependent activation pathway may involve a membrane-associated, tissue-specific protein kinase that phosphorylates the channel (or a regulatory protein that controls channel activity). This pathway could be interrupted because there is a defect in the cAMP-dependent kinase, or because the channel (or regulatory protein) cannot serve as an appropriate substrate for phosphorylation. Clearly, a better understanding of the molecular mechanisms that interpret the cAMP signal is needed.

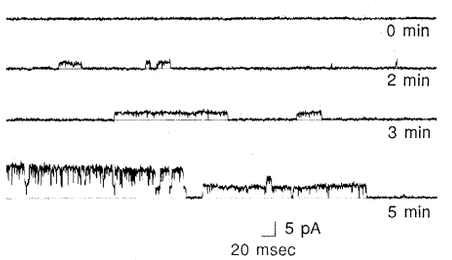

Fig. 5. Activation of chloride channel activity in a cell-attached patch from a CF cell by addition of 2 μ*M* A23187 to the bath at the beginning of the experiment (0 min). These data are typical of seven experiments on cells from three CF patients and three experiments on cells from two controls. Cells were bathed in the modified Ringer solution containing 1 m*M* Ca^{2+}. The holding potential was +60 mV throughout.

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4. Experiments were conducted at room temperature in a 35-mm dish equipped for solution change by a push-pull syringe system. Cells were visualized with Hoffmann modulation optics, and cell-attached and inside-out patches were formed as described (*13*). Unitary currents were amplified by a List EPC-7 voltage clamp and recorded on FM tape. Analyses were performed with an Indec 11/73 system, and the records were copied with a Hewlett-Packard X-Y plotter (model 7475A). Experimental solutions were buffered to *p*H 7.2 with 10 m*M* Hepes. The pipette solution was 150 m*M* NaCl and 2 m*M* $CaCl_2$. Bath solutions were a modified Ringer (140 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$) or Ca^{2+}-EGTA–buffered solutions (150 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* EGTA, and sufficient $CaCl_2$ to obtain free Ca^{2+} concentrations in the range 20 n*M* to 10 μ*M*) (*14*).
5. Cell culture was as described (*6*) with the following changes. Tracheal segments were obtained within 24 hours post-mortem and incubated for 20 minutes in Ringer solution containing 1 m*M* dithiothreitol (DTT) to solubilize mucins; 1 m*M* DTT was also included in the dissociation solutions. These solutions also contained tobramycin (10 μg/ml) and mezlocillin (100 μg/ml). Cells were plated at a density of 2.5×10^4 cells per milliliter and fed twice weekly with a mixture of Dulbecco's modified Eagle's medium and F12 (1:1) supplemented with newborn calf serum (5%), insulin (10 μg/ml), and hydrocortisone (5 μg/ml). The patch-clamp experiments were carried out with primary cultures or first passage cells and these were used for up to 1 week after plating.
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