

# cAMP-activated Apical Membrane Chloride Channels in *Necturus* Gallbladder Epithelium

## *Conductance, Selectivity, and Block*

J. COPELLO, T. A. HEMING, Y. SEGAL, and L. REUSS

From the Departments of Physiology and Biophysics and Internal Medicine, University of Texas Medical Branch, Galveston, Texas 77555

**ABSTRACT** Elevation of intracellular cAMP levels in *Necturus* gallbladder epithelium (NGB) induces an apical membrane  $\text{Cl}^-$  conductance ( $G_{\text{Cl}}^{\text{a}}$ ). Its characteristics (i.e., magnitude, anion selectivity, and block) were studied with intracellular microelectrode techniques. Under control conditions, the apical membrane conductance ( $G^{\text{a}}$ ) was  $0.17 \text{ mS}\cdot\text{cm}^{-2}$ , primarily ascribable to  $G_{\text{K}}^{\text{a}}$ . With elevation of cell cAMP to maximum levels,  $G^{\text{a}}$  increased to  $6.7 \text{ mS}\cdot\text{cm}^{-2}$  and became anion selective, with the permeability sequence  $\text{SCN}^- > \text{NO}_3^- > \text{I}^- > \text{Br}^- > \text{Cl}^- \gg \text{SO}_4^{2-} \sim \text{gluconate} \sim \text{cyclamate}$ .  $G_{\text{Cl}}^{\text{a}}$  was not affected by the putative  $\text{Cl}^-$  channel blockers  $\text{Cu}^{2+}$ , DIDS, DNDS, DPC, furosemide, IAA-94, MK-196, NPPB, SITS, verapamil, and glibenclamide. To characterize the cAMP-activated  $\text{Cl}^-$  channels, patch-clamp studies were conducted on the apical membrane of enzyme-treated gallbladders or on dissociated cells from tissues exposed to both theophylline and forskolin. Two kinds of  $\text{Cl}^-$  channels were found. With  $\sim 100 \text{ mM Cl}^-$  in both bath and pipette, the most frequent channel had a linear current-voltage relationship with a slope conductance of  $\sim 10 \text{ pS}$ . The less frequent channel was outward rectifying with slope conductances of  $\sim 10$  and  $20 \text{ pS}$  at  $-40$  and  $40 \text{ mV}$ , respectively. The  $\text{Cl}^-$  channels colocalized with apical maxi- $\text{K}^+$  channels in 70% of the patches. The open probability ( $P_o$ ) of both kinds of  $\text{Cl}^-$  channels was variable from patch to patch (0.3 on average) and insensitive to  $[\text{Ca}^{2+}]$ , membrane voltage, and pH. The channel density ( $\sim 0.3/\text{patch}$ ) was one to two orders of magnitude less than that required to account for  $G_{\text{Cl}}^{\text{a}}$ . However, addition of 250 U/ml protein kinase A plus 1 mM ATP to the cytosolic side of excised patches increased the density of the linear 10-pS  $\text{Cl}^-$  channels more than 10-fold to four per patch and the mean  $P_o$  to 0.5, close to expectations from  $G_{\text{Cl}}^{\text{a}}$ . The permeability sequence and blocker insensitivity of the PKA-activated channels were identical to those of the apical membrane. These data strongly suggest that 10-pS  $\text{Cl}^-$  channels are responsible for the cAMP-induced increase in apical membrane conductance of NGB epithelium.

Address correspondence to Dr. Luis Reuss, Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77555-0641.

## INTRODUCTION

Fluid absorption by *Necturus* gallbladder (NGB) epithelium is inhibited by elevation of the intracellular cAMP concentration ( $[cAMP]_i$ ) (Petersen and Reuss, 1983; Reuss, Segal, and Altenberg, 1991). The reduction in fluid absorption results from concurrent inhibition of apical membrane  $Na^+H^+$  and  $Cl^-HCO_3^-$  exchangers (Reuss and Petersen, 1985; Reuss, 1987) and activation of apical membrane  $Cl^-$  and  $K^+$  conductances ( $G_{Cl}^a$  and  $G_K^a$ , respectively; Petersen and Reuss, 1983; Cotton and Reuss, 1991). In NGB epithelium bathed in  $HCO_3^-/CO_2$ -buffered solutions, both apical and basolateral membranes are highly  $K^+$  selective and the apparent ratio of cell membrane resistances (apical/basolateral,  $R_a/R_b$ ) is  $\sim 5$  (for review, see Reuss, 1989). The basolateral membrane is  $Cl^-$  conductive, but no electrodiffusive  $Cl^-$  permeability is found in the apical membrane (Petersen and Reuss, 1983; Stoddard and Reuss, 1989). When  $[cAMP]_i$  is maximally elevated, the cell membranes depolarize by  $\sim 20$  mV and  $R_a/R_b$  falls to  $< 0.10$  (Duffey, Hainau, Ho, and Bentzel, 1981; Petersen and Reuss, 1983). These effects are primarily the consequence of activation of  $G_{Cl}^a$ , which becomes the dominant electrodiffusive pathway of the membrane. The properties of the cAMP-activated conductance (e.g., anion selectivity, pharmacological block) are not known. Moreover, there is little information about the cAMP-activated  $Cl^-$  pathway at the single-channel level. An anion channel has been purified from NGB epithelial cells and reconstituted in lipid bilayers (Finn, Tsai, and Falk, 1989; Tsai, Dillard, Rosenberg, Falk, Gaido, and Finn, 1991). The reconstituted channel (relative mass 219 kD) is anion selective and displays complex, multi-state channel behavior with a unitary conductance of  $\sim 62$  pS. In a recent publication, Finn, Dillard, and Gaido (1993) report that this same protein can also display a low conductance ( $\sim 9$  pS).

In this paper, we assessed (a) the magnitude of the  $Cl^-$  conductance by intracellular cable analysis, (b) the anion selectivity of the pathway, and (c) the pathway's sensitivity to agents reported to block  $Cl^-$  channels in other epithelia. Intracellular microelectrode and patch-clamp techniques were used in parallel, enabling a detailed comparison between the channel's macroscopic properties in intact epithelium and its microscopic features in cell-attached or excised membrane patches. These two approaches yielded similar results for the pathway's anion selectivity and insensitivity to blockers. A channel with a unitary conductance of  $\sim 10$  pS appears to be responsible for the cAMP-activated  $G_{Cl}^a$  in NGB epithelium. As observed for other cAMP-sensitive epithelial  $Cl^-$  channels (see reviews by McCann and Welsh, 1990; Reeves and Andreoli, 1992), the channel could be activated by exposure of the cytosolic surface of excised patches to protein kinase A (PKA) in the presence of cAMP and ATP.

Portions of this work have been published in preliminary form (Segal and Reuss, 1989; Heming, Copello, and Reuss, 1992).

## METHODS

Mudpuppies (*Necturus maculosus*) were obtained from NASCO (Ft. Atkinson, WI) or Kons Scientific Co., Inc. (Germantown, WI), kept in tap water at  $5^\circ C$ , and anesthetized by immersion in a 1 g/liter solution of tricaine methanesulfonate. All experiments were conducted at room

temperature ( $23 \pm 1^\circ\text{C}$ ). The excised tissues were usually bathed with a solution, hereafter called  $\text{HCO}_3^-$  physiological salt solution ( $\text{HCO}_3^-$ -PSS), containing (mM) 90 NaCl, 10  $\text{NaHCO}_3$ , 2.5 KCl, 1.8  $\text{CaCl}_2$ , 1.0  $\text{MgCl}_2$ , and 0.5 Na-phosphate, equilibrated with 1%  $\text{CO}_2$ /balance air (pH 7.65). In anion selectivity studies, we used a HEPES-buffered solution (hereafter called HEPES-PSS) containing (mM) 97.5 NaCl, 2.5 KCl, 1.0  $\text{CaCl}_2$ , 1.0  $\text{MgCl}_2$ , and 10 HEPES (pH 7.4).

In most cases, the tissue preparations were studied under control conditions and after elevation of [cAMP]. The latter condition was achieved by exposure of tissues to 1 mM 8-Br-cAMP or to 5  $\mu\text{M}$  forskolin and 3.3 mM theophylline. Theophylline was always added to the basolateral bathing solution; 8-Br-cAMP and forskolin were added to either bathing solution. All of these protocols result in a maximal, stable activation of  $G_{\text{Cl}}^{\text{a}}$  (Petersen and Reuss, 1983; Reuss and Petersen, 1985; Reuss, 1987; Cotton and Reuss, 1991). Hereafter, we refer to tissues treated with 8-Br-cAMP, forskolin, or theophylline as "stimulated."

#### *Intracellular Microelectrode Techniques*

Excised gallbladders were mounted apical side up in a modified Ussing chamber. The transepithelial voltage ( $V_{\text{ms}}$ ), cell membrane voltages (apical,  $V_{\text{mc}}$ ; basolateral,  $V_{\text{cs}}$ ), transepithelial resistance ( $R_t$ ), and apparent cell membrane resistance ratio ( $R_a/R_b$ ) were measured as previously described (see review by Altenberg, Copello, Cotton, Dawson, Segal, Wehner, and Reuss, 1990). Single-barrel microelectrodes were prepared from borosilicate glass (1 mm o.d., 0.5 mm i.d.) with an inner glass fiber (Glass Company of America, Millville, NJ). The electrodes were filled with 3 M KCl and had resistances of 30–70  $\text{M}\Omega$  when immersed in  $\text{HCO}_3^-$ -PSS. Extracellular electrodes were an Ag-AgCl pellet in series with a  $\text{HCO}_3^-$ -PSS/agar bridge in the basolateral bathing solution and a flowing, saturated KCl bridge in series with a calomel half-cell in the apical bathing solution. This arrangement minimized changes in liquid junction potentials upon ionic substitutions. All ionic substitutions were isomolar. In microelectrode experiments, only the apical bathing solution was changed.

A two-point cable analysis was used to quantify the cell membrane resistances (Altenberg et al., 1990). Briefly, two cells were impaled with conventional microelectrodes. Hyperpolarizing current pulses (20 nA, 1-s duration, 3  $\cdot\text{min}^{-1}$  frequency) were passed through one electrode and the voltage deflection in a neighboring cell ( $\Delta V_x$ , where  $x$  is the distance separating the electrodes) was measured with the second intracellular microelectrode. Concomitantly,  $R_t$  and  $R_a/R_b$  were calculated at 20-s intervals from the voltage deflections elicited by transepithelial current pulses (50  $\mu\text{A} \cdot \text{cm}^{-2}$ , 1-s duration, 3  $\cdot\text{min}^{-1}$  frequency) with appropriate corrections for series resistances. The inter-electrode distance was varied to obtain a range of  $\Delta V_x$  values. The results from several tissues were pooled and the control values of  $\Delta V_x$  were plotted assigning to each of them an  $x$  value such that all points were perfectly fit by a Bessel function with a characteristic amplitude ( $A$ ) of 2.09 mV and space constant ( $\lambda$ ) of 269  $\mu\text{m}$  (see below). These are the average values for control tissues in  $\text{HCO}_3^-$ -PSS (Stoddard and Reuss, 1988). The experimental  $\Delta V_x$  values (e.g., stimulated tissues) were plotted in the same graph, each against its preassigned  $x$  value. The Bessel function  $K_0$  (Frömter, 1972; Reuss and Finn, 1975a) was then fit to the experimental data, according to

$$\Delta V_x = A \cdot K_0(x/\lambda) \quad (1)$$

$A$  and  $\lambda$  were obtained from the best fit, and the experimental value of  $R_z$  (i.e., the equivalent resistance to  $R_a$  and  $R_b$  in parallel;  $R_z = R_a \cdot R_b / (R_a + R_b)$ ) was then calculated from

$$R_z = 2\pi A \lambda^2 / i_0 \quad (2)$$

where  $i_o$  is the applied intracellular current.  $R_a$ ,  $R_b$ , and  $R_s$  (where  $R_s$  is the paracellular resistance) were calculated from  $R_a$ ,  $R_b$ , and  $R_a/R_b$  as previously described (Frömter, 1972; Reuss and Finn, 1975a).

### *Patch-Clamp Techniques*

The application of patch-clamp methods to NGB epithelium has been described previously (Altenberg et al., 1990; Segal and Reuss, 1990a). Briefly, excised gallbladders were pinned apical side up in a Sylgard-coated Petri dish and bathed with HEPES-PSS. Two preparations were utilized: intact epithelium and enzymatically dissociated cells. Intact NGB was stretched and folded apical side out over the top surface of a plastic block and held at the base with a plastic-coated wire clip. This allowed access to the folded region of the apical surface, which was somewhat easier to patch. The tissue surface was washed for 10 min with a stream of HEPES-PSS containing 1 mg/ml hyaluronidase. Dissociated cells were prepared by a 7-min incubation of scraped epithelial sheets in 1 mg/ml hyaluronidase, followed by centrifugation and resuspension of the cells in hyaluronidase-free HEPES-PSS. Hyaluronidase had no effect on membrane voltages or conductances either before or after elevation of  $[cAMP]_i$  (data not shown; see also Segal and Reuss, 1990a).

Patch-clamp pipettes were made of soda glass (blue-tip hematocrit capillary tubes; Fisher Scientific Co., Pittsburgh, PA) or borosilicate glass (TW150-6; World Precision Instruments, Inc., Sarasota, FL). The pipettes were pulled with a two-stage vertical puller (model PP-103; Narishige, Tokyo, Japan) and coated at the tip with Sylgard 184 (Dow Corning Corp., Midland, MI). Borosilicate glass pipettes were fire-polished under microscopic observation ( $\times 400$ ) and had electrical resistances of 8–10 M $\Omega$  when filled with standard solutions (see below). Filling solutions contained (mM) 100 KCl, NaCl or *N*-methyl-*D*-glucamine chloride (NMDG-Cl), 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 10 HEPES (pH 7.4). In some experiments, 250 U/ml PKA, 250  $\mu$ M cAMP, and 1 mM ATP were added to the pipette and/or bath solutions.

Patch pipettes were mounted in a holder connected to the headstage of the patch-clamp amplifier (see below). The reference electrode was a PSS-agar bridge connected to an Ag-AgCl wire. Gigaohm seals were obtained by lightly touching cells with a pipette under microscopic observation and applying gentle suction. Excision by tapping the microscope stage yielded inside-out patches in approximately one-third of the attempts. Vesicles formed frequently; disruption by application of extreme holding voltages yielded inside-out or outside-out patches (see Altenberg et al., 1990). Chloride channels were identified by their high Cl<sup>-</sup>/gluconate selectivity and their reversal potential when exposed to solutions with different [NaCl] (isosmotic replacement with sucrose). Determination of excised patch orientation was facilitated by the high percentage of patches coexpressing Cl<sup>-</sup> and maxi-K<sup>+</sup> channels. Patch orientation was ascertained by one or more of the following criteria: (a) sensitivity of maxi-K<sup>+</sup> channels to cytosolic side [Ca<sup>2+</sup>] (Segal and Reuss, 1990a); (b) sensitivity of maxi-K<sup>+</sup> channels to extracellular tetraethylammonium (TEA<sup>+</sup>) (Segal and Reuss, 1990b); (c) sensitivity of Cl<sup>-</sup> channels to cytosolic side PKA (see Results).

Single-channel currents were measured with a List EPC-7 patch clamp (List Electronic, Darmstadt-Eberstadt, Germany) or an Axopatch 200 patch clamp (Axon Instruments, Inc., Foster City, CA) and stored on videotape using an Indec IR-2 digital instrument recorder (Indec Systems, Inc., Sunnyvale, CA). Representative recordings of 30–150-s duration were filtered at 500 Hz with an eight-pole Bessel filter (Frequency Devices, Inc., Haverhill, MA) and digitized at 2 kHz using a model DT2801 analog-to-digital conversion system (Data Translation, Inc., Marlboro, MA) for analysis by microcomputer. Data acquisition and analysis were carried out with the program PAT V6.1 (generously supplied by Dr. J. Dempster, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, Scotland). Single-channel

open probability ( $P_o$ ) was estimated from

$$P_o = (1/N) \sum_{i=1}^N iP_i \quad (3)$$

where  $i$  is a summation index,  $N$  is the number of channels in the patch (assumed to be equal to the maximum number of simultaneous open events), and  $P_i$  is the fraction of time during which  $i$  channels are open. This expression assumes that the channels gate identically and independently. It is possible that our estimates of  $P_o$  are biased in cases of very low  $P_o$  because the number of channels is difficult to determine, and in the case of a large number of channels in the patch because in this condition  $N$  is also difficult to assess (Colquhoun and Hawkes, 1983). For these reasons, more detailed kinetic studies were carried out in patches with apparently one channel (see Results).

Membrane voltages ( $V_m$ ) are reported with respect to the extracellular side of the patch, corrected for liquid-junction potentials. The latter were estimated from the voltage clamps between the reference electrode and a saturated KCl flowing junction, elicited by appropriate ionic substitutions. Currents depicted as positive (upward deflections) denote cation flux from intra- to extracellular compartments (anion flux in the opposite direction).

#### *Drugs and Chemicals*

Diphenylamine-2-carboxylic acid (DPC) was from Fluka Chemie AG (Buchs, Switzerland). The 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) was kindly provided by Dr. R. Greger (Physiologisches Institut, Albert-Ludwigs-Universität, Freiburg, Germany) and Dr. B. E. Pearce (Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX). The (6,7-dichloro-2-methyl-1-oxo-2-phenyl-5-indanyloxy) acetic acid (MK-196) was a gift from Merck, Sharp, and Dohme (West Point, PA). R(+)-[6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl]-oxy] acetic acid (IAA-94) and glibenclamide were from Research Biochemicals Inc. (Natick, MA). All other drugs and chemicals were from Sigma Chemical Co. (St. Louis, MO).

#### *Statistics and Curve Fitting*

Experimental values are expressed as mean  $\pm$  SEM. Curve fitting was done by nonlinear regression analysis of pooled data, using commercially available programs (Sigmaplot 5.1; Jandel Scientific, Corte Madera, CA; NFIT, Island Products, Galveston, TX).

## RESULTS

#### *Intracellular Microelectrode Studies*

*cAMP-activated apical membrane Cl<sup>-</sup> conductance.* Under control conditions, the apical membrane of NGB epithelial cells is primarily K<sup>+</sup> conductive (Reuss, 1989). In control tissues incubated in HCO<sub>3</sub><sup>-</sup>-PSS, lowering the [Cl<sup>-</sup>] of the apical bathing solution causes a slow hyperpolarization, probably due to an increase in basolateral membrane Cl<sup>-</sup> equilibrium potential ( $E_{Cl}$ ), a decrease in electrodiffusive Cl<sup>-</sup> permeability ( $P_{Cl}$ ), and an increase in relative  $P_K$  (Petersen and Reuss, 1983; Reuss and Costantin, 1984; Reuss, 1987); when the bathing solutions are buffered with 10 mM HEPES, lowering the [Cl<sup>-</sup>] of the apical bathing solution causes minimal changes in membrane voltages. In contrast, with either buffer, the same ion substitution after elevation of [cAMP]<sub>i</sub> elicits membrane voltage and conductance changes consistent

with a  $\text{Cl}^-$ -selective apical membrane (Petersen and Reuss, 1983; Reuss, 1987). Fig. 1 shows representative traces of the electrophysiological effects of reducing apical solution  $[\text{Cl}^-]$  in HEPES-PSS under control conditions and after elevation of  $[\text{cAMP}]_i$ .

*Effects of cAMP on cell membrane resistances.*  $G_{\text{Cl}}^a$  was quantified using the two-point cable analysis technique (see Methods). Fig. 2 shows the data and Bessel function fits under control (unstimulated) conditions, at the steady-state effect of forskolin plus theophylline, and after removal of apical solution  $\text{Cl}^-$  (isosmotic replacement with cyclamate). The cell membrane resistances are shown in Table I.

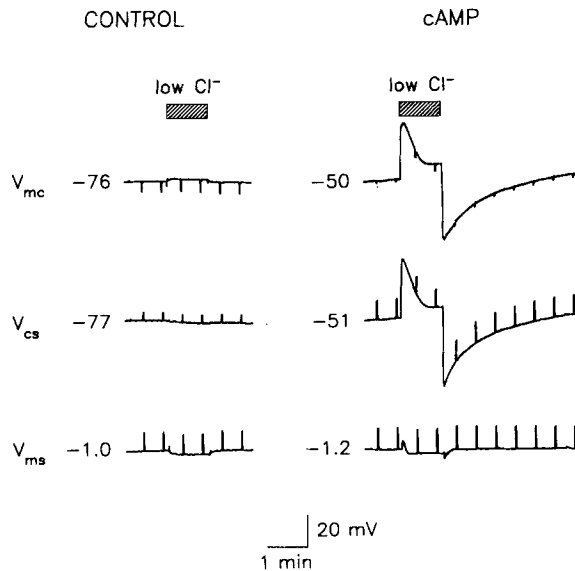


FIGURE 1. Effects of reductions in apical solution  $[\text{Cl}^-]$  on membrane voltages and resistances.  $V_{\text{mc}}$ ,  $V_{\text{cs}}$ , and  $V_{\text{ms}}$  are the apical, basolateral, and transepithelial voltages, respectively. Upward deflections denote apical solution-positive ( $V_{\text{ms}}$ ) or cell-positive ( $V_{\text{mc}}$ ,  $V_{\text{cs}}$ ) voltage changes. The brief voltage deflections were elicited by transepithelial constant current pulses and allowed for calculations of the transepithelial resistance ( $R_t$ ) and the apparent ratio of cell membrane resistances ( $R_a/R_b$ ). (Left) Apical solution  $[\text{Cl}^-]$  was reduced, for 1 min, from 104 to 10.4 mM ( $\text{Cl}^-$  replaced with cyclamate) in a

control tissue (i.e., without elevation of  $[\text{cAMP}]_i$ ). There are small changes in membrane voltages, mostly caused by a paracellular diffusion potential, and minor changes in  $R_t$  and  $R_a/R_b$ . (Right) Exposure to theophylline plus forskolin causes the following effects: depolarization of  $V_{\text{mc}}$  and  $V_{\text{cs}}$  by  $\sim 25$  mV, apical solution-negative change in  $V_{\text{ms}}$  by 0.2 mV, a small increase in  $R_t$ , and a dramatic fall in  $R_a/R_b$ . Upon lowering apical solution  $[\text{Cl}^-]$ , there is a rapid depolarization of both cell membranes followed by partial repolarization during the period in the low- $\text{Cl}^-$  solution, and a large, transient repolarization upon return to control solution. Note also that  $R_a/R_b$  is elevated during the period in low- $\text{Cl}^-$  apical bathing solution (see Petersen and Reuss, 1983).

The principal effects of cAMP were to decrease  $R_a$  from  $\sim 5,800$  to  $\sim 150 \Omega \cdot \text{cm}^2$  and  $R_z$  from  $\sim 900$  to  $\sim 140 \Omega \cdot \text{cm}^2$ . To ascertain that this effect was due to an increase in  $G_{\text{Cl}}^a$ , we removed apical solution  $\text{Cl}^-$ . Although this removal increased  $R_z$  to  $\sim 1,300 \Omega \cdot \text{cm}^2$ ,  $R_a/R_b$  did not recover fully. Hence, during elevation of  $[\text{cAMP}]_i$  and in  $\text{Cl}^-$ -free medium,  $R_a$  is lower and  $R_b$  is higher than the respective control values. At least in part, these results reflect activation of apical membrane maxi- $\text{K}^+$  channels because adding 5 mM TEA $^+$  to the  $\text{Cl}^-$ -free apical bathing solution further elevated  $R_a$  (data not shown). From the resistance values in  $\text{Cl}^-$  solution with maximal cAMP

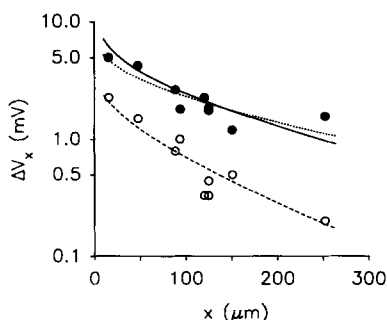


FIGURE 2. Intracellular cable analysis under control conditions (before elevation of [cAMP]<sub>i</sub>; *solid line*) and after stimulation with forskolin plus theophylline (*open circles, dashed line*), and upon Cl<sup>-</sup> removal after stimulation (*filled circles, dotted line*). ΔV<sub>x</sub> denotes the voltage deflection as a function of distance, x (see Methods). The data were obtained during continuous impalements across the apical membrane before and ~10 min after elevating [cAMP]<sub>i</sub>.

The curves are fits of the Bessel function  $K_0$  to the data (see Methods for details). The control curve is characterized by the parameters  $A = 2.09$  mV and  $\lambda = 269$  μm. Note that in every paired observation, ΔV<sub>x</sub> was less in stimulated tissues. Removing apical solution Cl<sup>-</sup> in high-cAMP preparations restored ΔV<sub>x</sub> to near control levels.

effect, we compute  $G_{Cl}^a = 6$  mS·cm<sup>-2</sup>, about twice the value calculated by Petersen and Reuss (1983).

*Anion selectivity of the cAMP-stimulated apical membrane conductance.* Anion selectivity of the apical membrane was studied under control conditions and after exposure to forskolin plus theophylline. The experiments were done in HEPES-PSS in order to compare with the patch-clamp results. Cl<sup>-</sup> was nominally removed from the apical bathing solution and replaced mole-by-mole with cyclamate (in the cases of NaCl, KCl, and CaCl<sub>2</sub>) or gluconate (in the case of MgCl<sub>2</sub>). Both control and stimulated preparations were exposed to Cl<sup>-</sup>-free solution. After membrane voltages and resistances reached steady-state values, 10 or 100 mM cyclamate was replaced with one of several anions (SCN<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, I<sup>-</sup>, Br<sup>-</sup>, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, or gluconate) for a 30-s period. The solution was exchanged at a rate of 30 ml/min and the volume of the apical compartment was 0.5 ml; hence, assuming ideal mixing, the time constant for exchange of the bulk solution was 1 s. The relative anion permeabilities of the apical membrane were estimated from the magnitude of the initial negative deflection in  $V_{mc}$  upon the ionic substitution and from the accompanying change in  $R_a/R_b$ .

TABLE I  
Effects of Cl<sup>-</sup> Removal from the Apical Bathing Solution on Membrane Resistances before and after Exposure to Theophylline and Forskolin

Condition	$R_a/R_b$	$R_t$	$R_a$	$R_b$
		$\Omega \cdot cm^2$	$\Omega \cdot cm^2$	$\Omega \cdot cm^2$
Unstimulated	$5.26 \pm 1.32$	$210 \pm 14$	5,816	1,106
Theophylline + forskolin	$0.07 \pm 0.01$	$238 \pm 17$	152	2,170
Theophylline + forskolin, Cl <sup>-</sup> free	$0.33 \pm 0.05$	$281 \pm 18$	1,712	5,187

Data are means ± SEM of nine paired experiments. Membrane resistance measurements and calculations were carried out as described in Methods.  $R_a/R_b$ , apparent ratio of apical and basolateral membrane resistances;  $R_t$ , transepithelial resistance;  $R_a$ , apical membrane resistance;  $R_b$ , basolateral membrane resistance.

Under control conditions (i.e., without elevation of  $[cAMP]_i$ ), apical  $Cl^-$  replacement with cyclamate produced a hyperpolarization that reached a plateau in  $\sim 2$  min. At 3 min,  $V_{mc}$  was  $-66 \pm 5$  mV, compared with  $-60 \pm 5$  in  $Cl^-$ -containing solution ( $n = 5$ ,  $P < 0.05$ ).  $R_t$  increased from  $151 \pm 18$  to  $197 \pm 14 \Omega \cdot cm^2$  ( $P < 0.05$ ) and  $R_a/R_b$  did not change significantly ( $2.62 \pm 0.51$  and  $2.98 \pm 0.45$ , respectively). The changes in  $V_{mc}$  and  $R_a/R_b$  produced by the anion substitutions are illustrated in Fig. 3 and summarized in Table II.  $SCN^-$  and  $NO_3^-$  produced the greatest changes in  $V_{mc}$  and  $R_a/R_b$  during partial (10 mM) substitutions of cyclamate.  $V_{mc}$  also hyperpolarized significantly, but much less so, with  $Cl^-$ ,  $Br^-$ , and  $I^-$ . No significant changes in  $R_a/R_b$  were observed with the latter anions. When 10 mM cyclamate was replaced with  $SO_4^{2-}$  or gluconate, neither  $V_{mc}$  nor  $R_a/R_b$  changed significantly.

In a similar series of experiments, 100 mM cyclamate was replaced with another anion.  $SCN^-$  and  $NO_3^-$  again elicited large changes in  $V_{mc}$  ( $\sim 10$  times those seen with 10-mM substitutions) and large changes in  $R_a/R_b$ .  $Cl^-$ ,  $Br^-$ ,  $I^-$ , gluconate, and sulfate

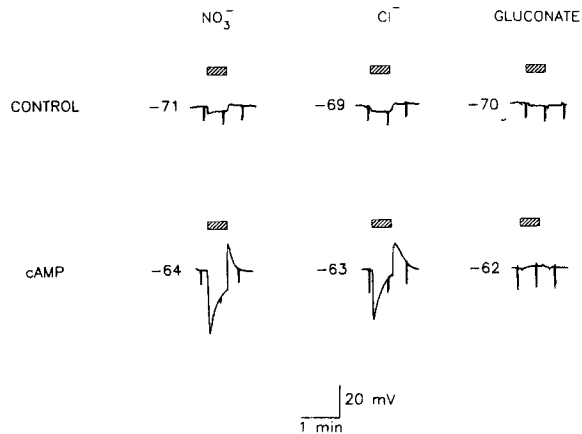


FIGURE 3. Anion selectivity of the apical membrane. Effects of partial replacement (10 mM) of apical  $Cl^-$ -free (cyclamate) solution with  $NO_3^-$ ,  $Cl^-$ , or gluconate in control and cAMP-stimulated preparations.  $V_{mc}$  records are shown. Under control conditions, replacing 10 mM cyclamate with  $NO_3^-$ ,  $Cl^-$ , or gluconate on the apical side had small effects on  $V_{mc}$ . In contrast, after elevating  $[cAMP]_i$ , large cell negative transients were observed with  $NO_3^-$  and  $Cl^-$ , but not with gluconate. See Table II and text.

had no significant effects on  $V_{mc}$  or  $R_a/R_b$ . These results suggest that, of the anions used, only  $NO_3^-$  and  $SCN^-$  permeate the apical membrane of unstimulated tissues via electrodiffusive pathways.

In tissues stimulated with forskolin plus theophylline,  $Cl^-$  removal (cyclamate replacement) caused a rapid depolarization of the apical membrane followed by a slow repolarization (Fig. 1) which reached a steady state in 2–3 min. The steady-state values for stimulated tissues in  $Cl^-$ -containing solution were  $V_{mc} = -59 \pm 4$  mV,  $R_a/R_b = 0.03 \pm 0.02$ , and  $R_t = 274 \pm 30 \Omega \cdot cm^2$ . In cyclamate solution, the values were  $V_{mc} = -59 \pm 4$  mV,  $R_a/R_b = 0.86 \pm 0.08$ , and  $R_t = 400 \pm 42 \Omega \cdot cm^2$ . Both  $R_a/R_b$  and  $R_t$  in cyclamate were significantly different from values in  $Cl^-$ -containing solution ( $n = 5$ ,  $P < 0.01$ ). Partial (10 mM) replacement of cyclamate with  $SCN^-$ ,  $NO_3^-$ , or halogen anions produced a rapid hyperpolarization of the apical membrane in cAMP-activated tissues. The magnitude of the hyperpolarization was  $\sim 10$ -fold greater than that observed in control tissues. As depicted in Table II, the magnitudes



of the  $V_{mc}$  changes in stimulated tissues were  $SCN^- > NO_3^- > I^- \sim Br^- > Cl^-$ . The substitutions also yielded significant decreases in  $R_a/R_b$ , with the sequence  $SCN^- > NO_3^- > Br^- \sim Cl^- \sim I^-$ . The latter results are less reliable than the  $V_{mc}$  data because  $R_a/R_b$  was measured at  $\sim 20$  s after the solution change; at this time, there are significant changes in cell volume and ionic composition (Petersen and Reuss, 1983; Reuss, 1987; Cotton and Reuss, 1991).

To quantify anion selectivity of the cAMP-activated pathway, we start from the Goldman-Hodgkin-Katz membrane voltage equation. This is based on the good fits of this kind of formulation to single-channel data (see below). We assume that only  $K^+$ ,  $Cl^-$ , and test anions ( $A^-$ ) are permeant. Cyclamate is assumed to be impermeant. Further, we assume that  $P_K$  and  $[K^+]_i$  are constant. Then, the anionic permeability

TABLE II  
Changes in Apical Membrane Voltage ( $V_{mc}$ ) and Zero Current Voltage ( $E_a$ ), and  $R_a/R_b$  Produced by Replacing 10 mM Cyclamate with Another Anion in the Apical Bathing Solution, before (Control) and after Exposure to Theophylline and Forskolin (cAMP)

Anion	$\Delta V_{mc}$		$\Delta(R_a/R_b)$		$\Delta E_a$ cAMP
	Control	cAMP	Control	cAMP	
	<i>mV</i>				<i>mV</i>
SCN <sup>-</sup>	-5.9 ± 0.9*	-56.7 ± 3.6*	-1.02 ± 0.31*	-0.80 ± 0.05*	-60
NO <sub>3</sub> <sup>-</sup>	-4.7 ± 0.7*	-48.4 ± 4.1*‡	-0.53 ± 0.33	-0.77 ± 0.09*	-50
I <sup>-</sup>	-2.2 ± 0.3*‡	-41.7 ± 2.7*‡	-0.08 ± 0.08	-0.68 ± 0.06*	-48
Br <sup>-</sup>	-2.5 ± 0.3*	-41.6 ± 1.7*	0.00 ± 0.02	-0.65 ± 0.04*	-46
Cl <sup>-</sup>	-3.0 ± 0.3*	-37.8 ± 2.1*‡	0.03 ± 0.01	-0.62 ± 0.04*	-40
SO <sub>4</sub> <sup>2-</sup>	1.1 ± 0.4‡	-0.5 ± 0.4‡	0.02 ± 0.02	-0.06 ± 0.03	-2
Gluconate	-0.7 ± 0.4	0.0 ± 0.3	-0.02 ± 0.02	-0.07 ± 0.04	-2

$\Delta V_{mc}$  and  $\Delta(R_a/R_b)$  data are means ± SEM of five paired experiments.  $\Delta E_a$  values were calculated as explained in the text. The tissues were exposed to Na-cyclamate-PSS on the apical side before (control) and after exposure to forskolin and theophylline (cAMP).  $\Delta V_{mc}$  is the maximum change in apical membrane voltage upon substituting 10 mM cyclamate for the same concentration of the anion indicated;  $\Delta(R_a/R_b)$  is the change in apparent ratio of membrane resistances 20 s after the substitution;  $\Delta E_a$  is the estimated change in the zero current apical membrane voltage evoked by the ionic substitution. Values of  $V_{mc}$  and  $R_a/R_b$  in Na-cyclamate PSS before 10 mM Cl<sup>-</sup> are given in the text. \*Significantly different from 0; ‡significantly different from the preceding value in the same column ( $P < 0.05$ ).

ratios are approximated by:

$$P_A/P_{Cl} = \frac{1 - \exp(-\Delta E_A F/RT)}{1 - \exp(-\Delta E_{Cl} F/RT)} \quad (4)$$

where  $P_A$  and  $P_{Cl}$  are the electrodiffusive permeability coefficients for  $A^-$  and  $Cl^-$ , respectively,  $\Delta E_A$  and  $\Delta E_{Cl}$  are the magnitudes of the initial changes in apical membrane zero-current voltage ( $E_a$ ) elicited by replacement of cyclamate with  $A^-$  and  $Cl^-$ , respectively, and  $F$ ,  $R$ , and  $T$  have their usual meanings. The validity of Eq. 4 depends on assumptions pertaining to  $[K^+]_i$  and  $P_K$ , which are addressed below.

To estimate the changes in  $E_a$ , we carried out an equivalent circuit analysis (Reuss and Finn, 1975a, b). Several simplifications and assumptions were necessary: (a)  $R_b$  (obtained from cable analysis, Table I) was assumed to remain constant at all times; (b) the zero-current voltages of the cell membranes ( $E_a$  and  $E_b$ ) and paracellular pathway ( $E_s$ ) were estimated using conventional circuit analysis equations (Reuss and Finn, 1975a) for cAMP-stimulated tissues exposed to  $\text{Cl}^-$ -free medium on the apical side; (c)  $E_b$  was assumed to remain unchanged upon the apical solution ionic substitution; (d) contributions of changes in  $E_s$  and  $R_s$  were shown to be negligible; and (e) the change in  $R_a$  was estimated from the value of  $R_a/R_b$  obtained 20 s after the ionic substitution. A modified form of previously derived circuit analysis equations (Reuss and Finn, 1975a, b) was used to estimate  $E'_a$ , the zero-current voltage after the ionic substitution:

$$E'_a = \frac{R'_a + R_b + R'_s}{R_b + R'_s} \left[ \Delta V_{mc} + \frac{E_a(R_b + R_s) + R_a(E_b - E_s)}{R_a + R_b + R_s} - \frac{R'_a(E_b - E'_s)}{R'_a + R_b + R'_s} \right] \quad (5)$$

where ' denotes values after exposure to 10 mM test anion.

This analysis yielded the  $\Delta E_a$  values shown in the last column of Table II. Inserting these values into Eq. 4, the  $P_A/P_{\text{Cl}}$  values were  $\text{SCN}^- : \text{NO}_3^- : \text{I}^- : \text{Br}^- : \text{Cl}^- : \text{SO}_4^{2-} : \text{gluconate} = 2.50 : 1.61 : 1.47 : 1.34 : 1.00 : 0.02 : 0.02$ . Calculations of  $P_A/P_{\text{Cl}}$  from the raw  $\Delta V_{mc}$  data gave exactly the same sequence; individual values differed by 10% or less. Given the assumptions and simplifications listed above, we do not stress the numerical values of these permeability ratios. However, we use the apparent permeability sequence to compare with single-channel data.

A problem with the preceding analysis is that the change in  $V_{mc}$  elicited by exposure to a permeant anion is expected to change the  $P_o$  of maxi- $\text{K}^+$  channels present in the apical membrane (Segal and Reuss, 1990a). To examine this issue, we carried out a series of five experiments in which  $P_{\text{SCN}^-}/P_{\text{Cl}}$  and  $P_{\text{Br}^-}/P_{\text{Cl}}$  were determined in the absence and presence of 5 mM  $\text{TEA}^+$ . The values of  $\Delta V_{mc}$  in the absence of  $\text{TEA}^+$  were (in mV):  $-54 \pm 4$  for  $\text{SCN}^-$ ,  $-43 \pm 3$  for  $\text{Br}^-$ , and  $-41 \pm 3$  for  $\text{Cl}^-$  (all significantly different from each other,  $P < 0.005$ ), corresponding to  $P_{\text{SCN}^-}/P_{\text{Cl}} = 1.84$  and  $P_{\text{Br}^-}/P_{\text{Cl}} = 1.10$ . In  $\text{TEA}^+$ , the  $\Delta V_{mc}$  values (in mV) were  $-68 \pm 3$  for  $\text{SCN}^-$ ,  $-55 \pm 3$  for  $\text{Br}^-$ , and  $-52 \pm 3$  for  $\text{Cl}^-$ , corresponding to  $P_{\text{SCN}^-}/P_{\text{Cl}} = 2.01$  and  $P_{\text{Br}^-}/P_{\text{Cl}} = 1.15$ . These results indicate that activation of maxi- $\text{K}^+$  channels does not influence assessment of the anion selectivity of the apical membrane under our experimental conditions.

*Sensitivity of the  $\text{Cl}^-$  conductance to putative  $\text{Cl}^-$  channel blockers.* We have previously shown that the cAMP-activated  $\text{Cl}^-$  conductance of the apical membrane of NGB epithelium is insensitive to DPC and  $\text{Zn}^{2+}$  (Reuss, Costantin, and Bazile, 1987; Kitchens, Dawson, and Reuss, 1990). However, both agents appear to block the  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchanger. In this study we tested other putative  $\text{Cl}^-$  channel blockers (for review, see Cabantchik and Greger, 1992). To attain a high sensitivity, we measured the changes in  $V_{mc}$  elicited by changing apical solution  $[\text{Cl}^-]$  at a low level, i.e., from 2.5 to 12.5 mM, with and without blockers. Examples are shown in Fig. 4. As summarized in Table III, DPC, disulfonic stilbene derivatives (4,4'-diisothiocya-

natostilbene-2,2'-disulfonic acid [DIDS] and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid [SITS]), NPPB, DNDS, MK-196, and IAA-94, did not block the Cl<sup>-</sup> conductance. In addition, we tested verapamil, which inhibits Cl<sup>-</sup> currents presumably associated with P-glycoprotein in cancer cells (Valverde, Díaz, Sepúlveda, Gill, Hyde, and Higgins, 1992), and Cu<sup>2+</sup>, which inhibits Cl<sup>-</sup> transport in rat kidney brush-border membrane vesicles (Lipkowitz and Abramson, 1989). Both agents were ineffective. We also tested the effects of the above putative blockers on the  $V_{mc}$  change produced by lowering [Cl<sup>-</sup>] from 98 to 10 mM (data not shown). Again, none of the agents tested affected the response.

#### Patch-Clamp Studies

*Apical membrane Cl<sup>-</sup> channels are activated by cAMP in intact epithelium.* High-resistance seals are difficult to obtain in intact NGB epithelium (Segal and Reuss, 1990a). After

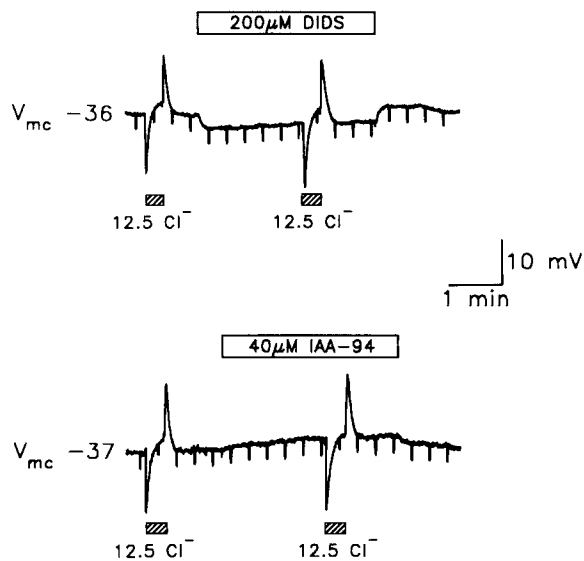


FIGURE 4. Apical membrane Cl<sup>-</sup> selectivity in cAMP-stimulated preparations in the absence of drugs (left part of each record) and in the presence of DIDS or IAA-94 (right part of each record). Protocol as in Fig. 3.  $V_{mc}$  was recorded in cyclamate-PSS for 2 min and then Cl<sup>-</sup> was raised from 2.5 to 12.5 mM for 30 s. Tissues were pretreated with theophylline plus forskolin. DIDS (200 μM) and IAA-94 (40 μM) were added to the apical bathing solution 2 min before measuring the response to 12.5 mM Cl<sup>-</sup>. Neither agent altered the  $V_{mc}$  change produced by raising [Cl<sup>-</sup>]; all other putative Cl<sup>-</sup> channel blockers tested were also ineffective (Table III).

treatment with hyaluronidase, gigaohm seals were obtained in ~3% of attempts. Addition of forskolin plus theophylline or 8-Br-cAMP to the bath activated Cl<sup>-</sup> channels in 3 of 33 high-resistance seals (Fig. 5). After excision of patches from stimulated tissues, Cl<sup>-</sup> channels were detected in 8 of the 33 seals. In five patches, the Cl<sup>-</sup> channels had a linear *I-V* relationship with  $11 \pm 1$  pS slope conductance.  $P_o$  was determined at both positive and negative voltages (30–60 mV) and found to be voltage independent.  $P_o$  was  $0.32 \pm 0.13$  (range 0.01–0.70). Channels in the remaining three patches exhibited outward rectification with conductances of  $21 \pm 2$  pS at 40 mV and  $12 \pm 2$  pS at -40 mV.  $P_o$  was  $0.21 \pm 0.10$  (range 0.06–0.42) and voltage independent. It is not entirely clear whether patch excision caused channel

TABLE III  
*Effects of Putative Cl<sup>-</sup> Channel Blockers on the Changes in V<sub>mc</sub> (ΔV<sub>mc</sub>) Produced by Elevating Apical Solution [Cl<sup>-</sup>] from 2.5 to 12.5 mM*

Agent	ΔV <sub>mc</sub>	
	Control	Agent
	<i>mV</i>	
Cu <sup>2+</sup> , 100 μM	-13.0 ± 1.3	-13.7 ± 1.1
DIDS, 200 μM	-12.7 ± 0.8	-12.7 ± 1.0
DNDS, 100 μM	-13.3 ± 0.9	-13.4 ± 0.4
DPC, 500 μM	-12.6 ± 0.5	-13.4 ± 1.0
Furosemide, 1 mM	-12.8 ± 0.8	-12.7 ± 0.7
IAA-94, 40 μM	-12.5 ± 0.4	-14.8 ± 1.0*
MK-196, 1 mM	-12.3 ± 0.5	-11.5 ± 0.6
NPPB, 100 μM	-13.6 ± 1.9	-15.5 ± 1.3
SITS, 200 μM	-13.7 ± 0.6	-14.5 ± 0.5
Verapamil, 100 μM	-11.7 ± 1.8	-15.8 ± 0.7*

Data are means ± SEM of five paired experiments. The period of exposure to 10 mM Cl<sup>-</sup> was 30 s. ΔV<sub>mc</sub> is the maximum change in apical membrane voltage upon substituting 10 mM cyclamate with Cl<sup>-</sup>. Exposure to agent was for 2 min before the Cl<sup>-</sup>-for-cyclamate substitution (see Fig. 6). \*Significantly different from the control value ( $P < 0.05$ ).

activation. It is also possible that vesicles formed in the cell-attached mode were broken upon excision.

*Cl<sup>-</sup> channels from dissociated gallbladder epithelial cells.* Because of the low yield of the above experiments, we studied the Cl<sup>-</sup> channel in cells dissociated after exposure to forskolin plus theophylline. Maxi-K<sup>+</sup> channels are observed in ~25% of patches. The presence of this channel, normally expressed in the apical but not in the basolateral membrane of the intact epithelium (Segal and Reuss, 1990a; Copello, Wehner, and Reuss, 1993), indicates that the apical membrane domain is present on the surface of dissociated cells. Cl<sup>-</sup> channels were observed in 43 of 226 gigaohm seals. When present, the average number of Cl<sup>-</sup> channels per patch was  $1.3 \pm 0.1$  (range 1–4). They colocalized with maxi-K<sup>+</sup> channels in ~70% of the experiments in which the solutions allowed for recording of both channels (Fig. 6).

In 30 of 43 patches, the Cl<sup>-</sup> channel had a linear *I-V* relationship (Fig. 7), with a conductance of  $11 \pm 0.2$  pS. As shown in Fig. 7, the reversal potential was 0 mV with (a) symmetrical NaCl solutions in the pipette and bath, (b) KCl and NaCl solutions in



FIGURE 5. Activation of Cl<sup>-</sup> channels in the apical membrane of intact epithelium by cAMP. Cell-attached patch held at  $V_p = 0$  mV. Zero-current levels are indicated to the right of the records. The record on the right starts 80 s after addition of forskolin plus theophylline to the apical bathing solution. In three experiments, single-channel currents were evident within ~3 min of drug addition. Pipette, NaCl solution; bath, NaCl-PSS; both were buffered with HEPES (pH 7.40).

tion of forskolin plus theophylline to the apical bathing solution. In three experiments, single-channel currents were evident within ~3 min of drug addition. Pipette, NaCl solution; bath, NaCl-PSS; both were buffered with HEPES (pH 7.40).

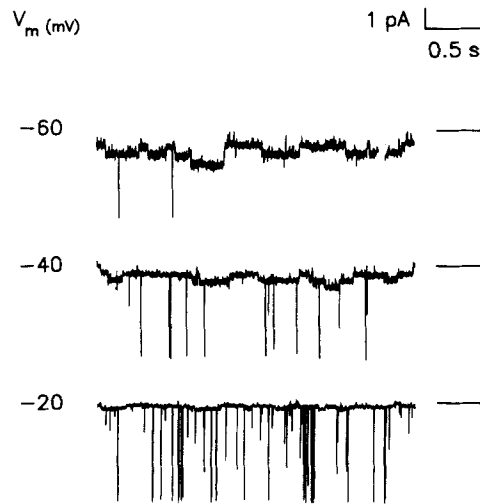


FIGURE 6. Colocalization of Cl<sup>-</sup> channels and maxi-K<sup>+</sup> channels in patches from dissociated cells. Pipette, KCl solution; bath, NaCl-PSS. Cl<sup>-</sup> channel and maxi-K<sup>+</sup> channel currents are depicted. V<sub>m</sub> are indicated on the left and zero-current levels on the right of the records. [Ca<sup>2+</sup>]<sub>i</sub> = 100 nM. Cl<sup>-</sup> channel currents are small (~0.6 pA at -60 mV) and exhibit slow kinetics; maxi-K<sup>+</sup> channel currents are larger (~12 pA at -60 mV) and appear as truncated spikes. The colocalization of these two kinds of channels is consistent with apical origin of the Cl<sup>-</sup> channels.

the pipette and bath, respectively, and (c) NMDGCl and NaCl solutions in the pipette and bath, respectively (data not shown). Reducing the NaCl concentration in the bath solution to half (isosmotic replacement with sucrose) shifted the reversal potential to about -15 mV, indicating near-ideal selectivity for Cl<sup>-</sup> over Na<sup>+</sup>. Upon replacement

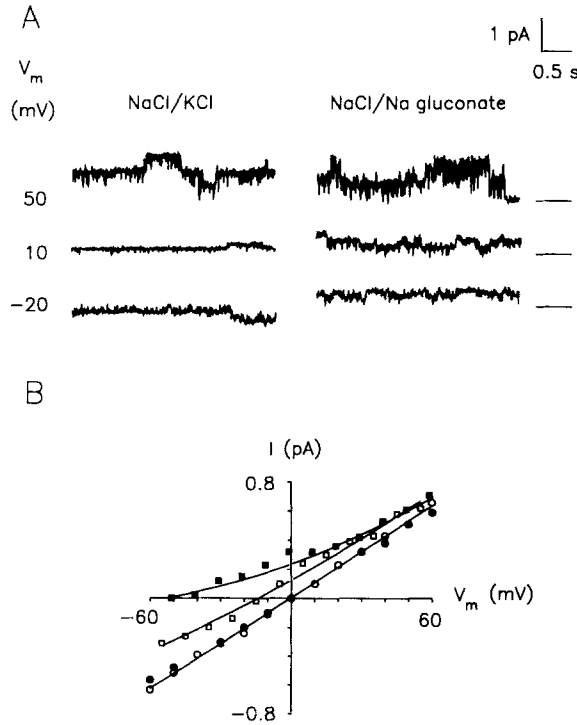


FIGURE 7. Linear Cl<sup>-</sup> channels in excised patches from cAMP-stimulated cells. (A) Single-channel events in an inside-out patch with NaCl- or Na gluconate-PSS in the bath. Pipette, KCl solution. Membrane voltages are indicated on the left and zero-current levels on the right of the current records. (B) *I-V* relationships of inside-out patches. The reversal potential in symmetrical Cl<sup>-</sup> was 0 mV with either NaCl (*open circles*) or KCl (*filled circles*) in the pipette solution. When [NaCl] was reduced to 50 mM (isosmotic sucrose replacement; *open squares*) the reversal potential was -15 mV, a value consistent with  $P_{Na}/P_{Cl} = 0.045$ . With gluconate in the bath (*filled squares*), the reversal potential was approximately -50 mV, which yields a permeability for

Cl<sup>-</sup> 12 times greater than that for gluconate. Lines depict fits of the Goldman-Hodgkin-Katz equation to the data. See Table IV.

of NaCl with Na-gluconate, the reversal potential shifted by  $\sim 50$  mV (Fig. 7), consistent with a permeability ratio (gluconate/Cl<sup>-</sup>) of 0.08 (compare Table IV).

Channel activity varied considerably from patch to patch. From experiments in which channel activity was recorded for at least 1 min at both positive and negative voltages, we calculated an average  $P_o$  of  $0.25 \pm 0.10$  ( $n = 8$ ). However, values ranged from near 0 to near 1. Within individual patches,  $P_o$  was independent of  $V_m$ . Channel conductance and  $P_o$  were unaffected by  $[Ca^{2+}]_i$  (in range of  $10^{-8}$ – $10^{-3}$  M) or the pH of either solution (in the range of 7.0–7.5). Cl<sup>-</sup> channel rundown was observed in 50% of excised patches in which records were maintained for at least 10 min.

To ascertain the reason for the variability of  $P_o$  values, we carried out kinetic analysis of three long-duration records in patches with apparently only one channel. The records were filtered at 500 Hz and digitized at 5 kHz and contained at least 2,000 events. The  $P_o$  values were 0.19, 0.62, and 0.92. Open-time distributions yielded good fits with two exponentials ( $\tau_1$  ranged from 0.9 to 1.7 ms and  $\tau_2$  ranged from 11.8 to 37.2 ms). The closed-time distributions were fit by one exponential in

TABLE IV  
*Anion Selectivity of the cAMP-activated Apical Membrane Cl<sup>-</sup> Channels*

Anion	$V_r$	$P_A/P_{Cl}$	$n$
	<i>mV</i>		
SCN <sup>-</sup>	$-14 \pm 4$	$1.86 \pm 0.26$	3
NO <sub>3</sub> <sup>-</sup>	$-13 \pm 2$	$1.74 \pm 0.17$	3
I <sup>-</sup>	$-10 \pm 2$	$1.51 \pm 0.10$	5
Br <sup>-</sup>	$-3 \pm 1$	$1.16 \pm 0.04$	4
Cl <sup>-</sup>	$0 \pm 0$	1	21
SO <sub>4</sub> <sup>2-</sup>	$30 \pm 2$	$0.18 \pm 0.02$	4
Gluconate	$46 \pm 3$	$0.12 \pm 0.02$	9
Cyclamate	$39 \pm 1$	$0.19 \pm 0.01$	4

Data shown are means  $\pm$  SEM;  $n$  = number of experiments  $V_r$ , reversal potential.  $P_A/P_{Cl}$  values were calculated from  $V_r$ , according to Eq. 6. See text.

the experiment with  $P_o$  0.92 ( $\tau = 0.4$  ms) and by two exponentials in the other two experiments: for  $P_o$  0.62,  $\tau_1 = 0.4$  and  $\tau_2 = 1.9$  ms; for  $P_o$  0.18,  $\tau_1 = 0.8$  and  $\tau_2 = 9.0$  ms. In other experiments, channel closures lasting for minutes were occasionally observed. These results suggest that the variability in the  $P_o$  values of the 10-pS Cl<sup>-</sup> channel is principally determined by the duration of the closed states, which vary considerably among patches. Smaller  $P_o$  values appear to result from the presence of one or more long-lasting closed states. A detailed kinetic analysis will be necessary for definitive conclusions.

In 13 of 43 experiments, the Cl<sup>-</sup> channels exhibited outward rectification (Fig. 8); the slope conductance was  $20 \pm 1$  pS at positive  $V_m$  and  $12 \pm 1$  pS at negative  $V_m$  ( $n = 13$ ). The gluconate/Cl<sup>-</sup> selectivity was  $0.10 \pm 0.04$  ( $n = 5$ ). Similar to the linear channels,  $P_o$  was highly variable from patch to patch and did not show voltage dependence. Steady-state changes in  $[Ca^{2+}]_i$  or in the pH of either solution (same ranges as above) had no effects on single-channel properties.

Pooling the results of experiments with intact epithelia and dissociated cells, the average number of Cl<sup>-</sup> channels per patch was  $\sim 0.3$ . From the macroscopic  $G_{Cl}^a$  (given by  $G_{Cl}^a = N \cdot P_o \cdot g$ , where  $g$  is the single-channel conductance), calculated from the cable analysis experiments ( $6 \text{ mS} \cdot \text{cm}^{-2}$ ) and the values of  $P_o$  (0.3) and  $g$  (10 pS) obtained from the patch-clamp experiments, we can estimate the value of  $N$  needed to account for  $G_{Cl}^a$ . The result (for our pipette tip size, the minimum patch surface area ranges from 0.8 to  $3.1 \mu\text{m}^2$ ) is 14–55 channels per patch, i.e., two orders of magnitude greater than the channel density detected. These results indicate that most of the cAMP-induced  $G_{Cl}^a$  is not expressed in patch-clamp experiments with intact epithelium or dissociated cells.

*Activation of Cl<sup>-</sup> channels by PKA + ATP.* To assess whether channels could be activated by phosphorylation, PKA and ATP were added to the pipette solution.

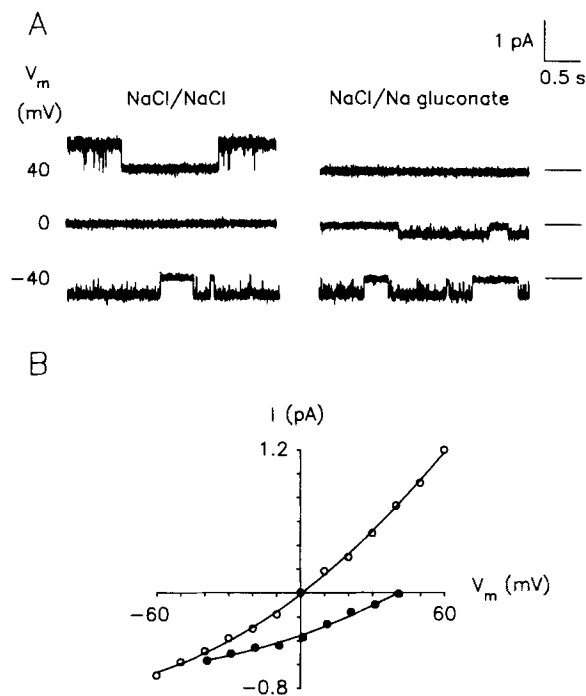


FIGURE 8. Rectifying Cl<sup>-</sup> channels in an outside-out patch from a cAMP-stimulated cell. (A) Single-channel events with NaCl- or Na gluconate-PSS in the bath. Pipette, NaCl solution. Membrane voltages are indicated on the left and zero-current levels on the right of each current record. (B)  $I$ - $V$  relationships. The reversal potential in symmetrical NaCl solution (*open circles*) was 0 mV. With gluconate in the bath (*filled circles*), the reversal potential was  $\sim 40$  mV.

When this treatment did not result in channel activation, PKA and ATP were also added to the bath. In preliminary experiments in which the orientation of the patch was established from TEA<sup>+</sup> sensitivity of maxi-K<sup>+</sup> channels, we found that PKA and ATP were effective only from the cytosolic side. Channel activation occurred in 5 of 10 attempts with 10–20 U/ml of catalytic subunit of PKA (dissolved in dithiothreitol solution). ATP alone activated Cl<sup>-</sup> channels in two additional patches. A remarkable increase in the number of channels per patch was observed with 250 U/ml PKA (plus ATP and cAMP), i.e., up to 12 current levels in a patch (Fig. 9). The reversal potential of the current was 0 mV with NMDGCl in the pipette and NaCl in the bath. Replacing bath Na<sup>+</sup> with K<sup>+</sup> had no effects on current amplitude or reversal

potential. In similar experiments,  $\text{Cl}^-$  selectivity was demonstrated by  $\text{Cl}^-$  replacement with gluconate in the bath. The PKA-activated channels had linear  $I$ - $V$  relationships, a slope conductance of  $10 \pm 0.3$  pS ( $n = 24$ ), and a voltage-independent  $P_o$  of  $0.49 \pm 0.06$  (range 0.25–0.81;  $n = 8$ ). The number of channels in outside-out patches with 250 U/ml PKA, 1 mM MgATP, and 0.25 mM cAMP in the pipette was  $4.1 \pm 0.7$  ( $n = 13$ ).  $N$  was similar ( $3.6 \pm 0.6$ ,  $n = 11$ ) in inside-out patches with the same concentrations of PKA, ATP, and cAMP in the bath. Channel rundown, assessed in records lasting  $>10$  min, was observed in 8 of 18 patches. Outward-rectifying  $\text{Cl}^-$  channels were not detected in these experiments. Although the channel density is still low compared with the estimate from the macroscopic conductance, the difference is less than in the preceding set of experiments (see Discussion).

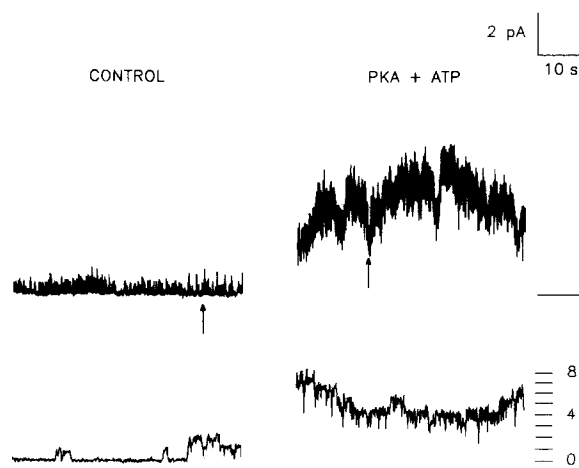


FIGURE 9. Activation of  $\text{Cl}^-$  channels by 250 U/ml of PKA plus 1 mM ATP. Single  $\text{Cl}^-$  channel events in an inside-out patch with NMDGCl solution in the pipette and NaCl-PSS in the bath. The preparation had been preexposed to forskolin and theophylline. Nominal free  $[\text{Ca}^{2+}]$  was 1 mM.  $V_m$  was  $-60$  mV; zero-current levels are indicated to the right of the records. Bottom panels depict time-expanded traces (1-s duration) starting at the places indicated by the arrows; the scale on the right denotes current levels.

current levels. Addition of PKA caused  $\text{Cl}^-$  channel activation. This record is representative of a total of 11 patches. With this PKA concentration, the mean number of channels per patch was approximately four.

*Anion selectivity of the 10-pS  $\text{Cl}^-$  channel.* The selectivity of  $\text{Cl}^-$  channels activated with PKA, ATP, and cAMP was investigated using inside-out and outside-out patches with HEPES-PSS (106 mM  $\text{Cl}^-$ ) in the pipette and anion-substituted solutions in the bath. Fig. 10 shows the mean  $I$ - $V$  relationships with  $\text{NO}_3^-$ ,  $\text{Cl}^-$ , or cyclamate in the bath. Table IV gives mean values for the relative permeabilities estimated from the reversal potentials. For monovalent anions, the following equation was used (modified from Hille, 1984):

$$V_r = \frac{RT}{F} \ln \frac{[\text{Cl}^-]_i}{[\text{Cl}^-]_o + (P_A/P_{\text{Cl}})[\text{A}^-]_o} \quad (6)$$

where  $V_r$  is the reversal potential,  $[\text{Cl}^-]$  and  $[\text{A}^-]$  are concentrations, the subscripts o and i denote bath and pipette solutions,  $P_A/P_{\text{Cl}}$  is the electrodiffusive



permeability ratio, and  $R$ ,  $T$ , and  $F$  have their usual meanings.  $[\text{Cl}^-]_o$  and  $[\text{Cl}^-]_i$  were 6 and 106 mM, respectively, and  $[\text{A}^-]_o$  was 100 mM. In the case of  $\text{SO}_4^{2-}$ , an appropriate derivation of the Goldman-Hodgkin-Katz equation was used.

The permeability sequence was  $\text{SCN}^- > \text{NO}_3^- > \text{I}^- > \text{Br}^- > \text{Cl}^- \gg \text{SO}_4^{2-} \sim \text{gluconate} \sim \text{cyclamate}$ . This result agrees with microelectrode assessments of the anion selectivity of the apical membrane upon stimulation with cAMP. For the above calculations, we assumed that the  $\text{Cl}^-$  channels are  $\text{Na}^+$  impermeable. This assump-

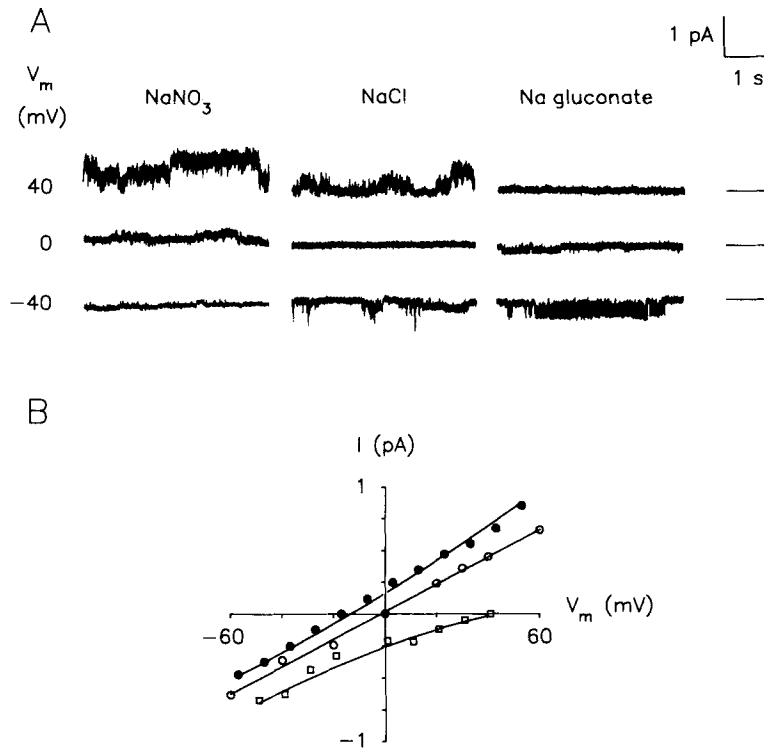


FIGURE 10. Anion selectivity of the cAMP-activated  $\text{Cl}^-$  channel. (A) Single  $\text{Cl}^-$  channel events in an outside-out patch with  $\text{NaNO}_3$ -PSS,  $\text{NaCl}$ -PSS, or  $\text{Na gluconate}$ -PSS in the bath. Pipette,  $\text{NaCl}$  solution.  $V_m$  is indicated on the left and zero-current levels on the right of the records. (B)  $I$ - $V$  relationships from the same patch. The reversal potentials were approximately  $-20$ ,  $0$ , and  $40$  mV for  $\text{NaNO}_3$ -PSS (open circles),  $\text{NaCl}$ -PSS (filled circles), or  $\text{Na gluconate}$ -PSS (open squares) in the bath, respectively.

tion is supported by the  $I$ - $V$  relationship obtained after replacing 50 mM  $\text{NaCl}$  of the bath solution with 100 mM sucrose. Fitting the Goldman-Hodgkin-Katz equation to the data yielded a  $P_{\text{Na}}/P_{\text{Cl}}$  of  $0.045 \pm 0.026$  ( $n = 4$ ,  $P > 0.05$ , not different from 0). Determination of the reversal potentials from linear interpolation was inappropriate in this case because of the curvature of the  $I$ - $V$  relationship.

**Blocker sensitivity of the 10-pS  $\text{Cl}^-$  channels.** Experiments with putative channel blockers were carried out on outside-out patches. The pipette was filled with

NMDGCl-PSS plus 250 U/ml PKA, 0.25 mM cAMP, and 1 mM ATP. In patches with active channels, current records were first obtained for at least 5 min in order to ensure stability of the channel activity. This precaution was necessary because channel rundown was common and could confound the assessment of blocker effect. Each blocker was tested in at least two experiments, at negative and positive  $V_m$ . Measurements were performed 1–5 min after addition of the agent and the channel activity was recorded for 1–2 min. Fig. 11 shows the lack of effect of DNDS. None of the blockers appreciably affected the channel current amplitudes. Only DIDS (200  $\mu$ M) decreased the  $P_o$  consistently, by merely  $16 \pm 2\%$  ( $n = 3$ ). In two experiments, SITS appeared to cause irreversible block, but in four experiments it had no effect. These results indicate that the external surface of the  $\text{Cl}^-$  channel has the same pattern of blocker insensitivity as  $G_{\text{Cl}}^a$ . Each agent listed in Table III was also tested using inside-out patches. Again, there were no measurable effects on channel activity. Glibenclamide, which blocks  $\text{Cl}^-$  channels from fibroblasts transfected with the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Sheppard and Welsh, 1992), had no effects on  $G_{\text{Cl}}^a$  or on single-channel events (two microelectrode experiments and one patch-clamp experiment, respectively).

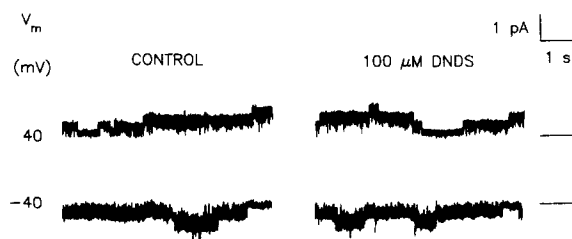


FIGURE 11. Lack of  $\text{Cl}^-$  channel block by 100  $\mu$ M DNDS in an outside-out patch held at  $V_m = -40$  or 40 mV. Zero-current level is indicated on the right. DNDS had no effects on single-channel currents or  $P_o$ .

#### DISCUSSION

cAMP increases dramatically the  $\text{Cl}^-$  conductance of the apical membrane of *Necturus* gallbladder epithelium (Petersen and Reuss, 1983). In this study we used the patch-clamp technique to identify the channels underlying the cAMP-induced  $G_{\text{Cl}}^a$ . We also used intracellular microelectrode methods to determine the magnitude of the  $\text{Cl}^-$  conductance, its anion selectivity, and its sensitivity to putative  $\text{Cl}^-$  channel blockers. These results were compared with those obtained in the patch-clamp experiments in order to characterize the conductance and the channels under similar experimental conditions. A major objective of our studies was to establish whether the  $\text{Cl}^-$  channels found in patch-clamp studies were in fact responsible for the membrane conductance. The main finding was a 10-pS  $\text{Cl}^-$  channel activated by PKA. This channel has properties entirely consistent with those of the cAMP-activated  $G_{\text{Cl}}^a$ . Hence, we conclude that this is the cAMP-activated apical membrane  $\text{Cl}^-$  channel. With less frequency, we found an outwardly rectifying channel of similar conductance and  $P_o$ . Its significance is uncertain (see below).

*Magnitude of the Apical Conductance under cAMP Stimulation*

To estimate the value of  $G^a$  after maximal elevation of  $[\text{cAMP}]_i$ , we carried out cable analysis experiments (see Methods and Results). cAMP caused a 40-fold increase in  $G^a$ , to  $6.6 \text{ mS}\cdot\text{cm}^{-2}$ .  $\text{Cl}^-$  removal from the apical bathing solution (cyclamate replacement) decreased  $G^a$  to  $0.58 \text{ mS}\cdot\text{cm}^{-2}$ , indicating that  $G_{\text{Cl}}$  accounts for at least 90% of  $G^a$ , in good agreement with earlier observations (Petersen and Reuss, 1983). Addition of the  $\text{K}^+$  channel blocker  $\text{TEA}^+$  to a  $\text{Cl}^-$ -free apical bathing solution decreased  $G^a$  further, to  $0.13 \text{ mS}\cdot\text{cm}^{-2}$ . Thus,  $\text{K}^+$  channels constitute the main conductive pathway remaining in the apical membrane in cAMP-stimulated tissues after  $\text{Cl}^-$  removal from the apical side. These data appear to rule out an apical membrane  $\text{HCO}_3^-$ -conductive pathway of the magnitude observed in guinea pig gallbladder (Stewart, Winterhager, Heintze, and Petersen, 1989). Previous results also argue against the possibility of a  $\text{HCO}_3^-$  conductance in the apical membrane of cAMP-stimulated NGB epithelial cells (Reuss, 1987).

*Anion Selectivity of the cAMP-stimulated Apical Cl<sup>-</sup> Conductance and the PKA-activated Cl<sup>-</sup> Channel*

The anion selectivity of the apical membrane conductance activated by cAMP was  $\text{SCN}^- > \text{NO}_3^- > \text{I}^- > \text{Br}^- > \text{Cl}^- \gg \text{SO}_4^{2-} \sim \text{gluconate} \sim \text{cyclamate}$ . The same sequence was demonstrated for the 10-pS  $\text{Cl}^-$  channels found in dissociated cells. The halogen anion sequence observed is opposite to the sequence of hydration energies ( $\text{Cl}^- > \text{Br}^- > \text{I}^-$ ; Wright and Diamond, 1977), which according to principles of equilibrium selectivity (Eisenman, 1962) suggests that the permeant anions interact with positively charged sites of low field strength.

*Comparison of Properties of the cAMP-stimulated Apical Cl<sup>-</sup> Conductance and the PKA-activated Cl<sup>-</sup> Channel*

A major conclusion of this work is that the 10-pS apical membrane  $\text{Cl}^-$  channel accounts for the cAMP-activated  $G_{\text{Cl}}^a$ . This conclusion is based on several lines of evidence. (a) As summarized above, the anion selectivity of the cAMP-activated  $\text{Cl}^-$  conductance and the  $\text{Cl}^-$  channel are the same. (b) Both  $G_{\text{Cl}}^a$  and  $\text{Cl}^-$  channels are insensitive to virtually every putative  $\text{Cl}^-$  channel blocker assayed (the sole positive result was with DIDS, which had a minor effect on  $P_o$  in the patch-clamp experiments). (c) The channels are activated by PKA. Assuming an area of  $1 \mu\text{m}^2$  per patch, and given that  $N = 4$ ,  $P_o = 0.5$ , and  $g = 10 \text{ pS}$  (for channels in patches treated with 250 U/ml of PKA) we compute  $G_{\text{Cl}}^a = 2 \text{ mS}\cdot\text{cm}^{-2}$ , which, given the difficulties inherent in both measurements, is not far from the values estimated in microelectrode experiments, i.e.,  $3\text{--}6 \text{ mS}\cdot\text{cm}^{-2}$  (Petersen and Reuss, 1983; this study). We point out that patches had as many as 12 active channels, which yields  $G_{\text{Cl}}^a$  values closer to the estimate from cable analysis data. It is unclear why  $\text{Cl}^-$  channel activity was much greater with PKA plus ATP treatment than with theophylline/forskolin treatment. In principle, the experimental technique could alter any step in the activation process. For instance, the proposed cytosolic  $\text{Cl}^-$  channel inhibitor (Krick, Disser, Hazama, Burckhardt, and Frömter, 1991; Kunzelmann, Tilmann, Hansen,

and Greger, 1991) might be increased by the experimental procedure, preventing channel activation in the cell-attached configuration; excision of the patch would result in loss of the inhibitor.

*Other Anion Channels in the Apical Membrane of Necturus Gallbladder Epithelium*

Outward rectification was observed in ~30% of the experiments in which channels were activated with theophylline and forskolin. However, this was not observed in excised patches activated by PKA, even when the number of channels was small. The explanation for these results is not clear. It could be that the rectifying channels are not activated by phosphorylation, or that the rectification is modulated by the degree of phosphorylation, being reduced with greater phosphorylation, as expected with PKA and ATP. The opposite effect, namely, disappearance of Cl<sup>-</sup> channel rectification by dephosphorylation, has been observed in A6 cells (Marunaka and Eaton, 1990). The ~62-pS channel purified by Finn and co-workers (Finn et al., 1989; Tsai et al., 1991) was never observed in our patch-clamp experiments.

*Comparison with Other Epithelial Cl<sup>-</sup> Channels*

Many Cl<sup>-</sup> channels have been characterized in epithelia (for reviews, see Gögelein, 1988; Frizzell and Halm, 1990; Anderson, Sheppard, Berger, and Welsh, 1992; Cabantchik and Greger, 1992; Fuller and Benos, 1992; Reeves and Andreoli, 1992). The cAMP-activated Cl<sup>-</sup> channels in secretory epithelia, thought to be CFTR, show some similarities with the cAMP-activated Cl<sup>-</sup> channels described here. As summarized in several recent reviews (Anderson et al., 1992; Fuller and Benos, 1992; Welsh, Anderson, Rich, Berger, Denning, Ostedgaard, Sheppard, Cheng, Gregory, and Smith, 1992), CFTR channels have linear or slightly outwardly rectifying *I-V* relationship and small conductance (5–12 pS). They are activated by PKA and the channel properties are independent of pH, intracellular [Ca<sup>2+</sup>], and membrane voltage. Furthermore, CFTR channels show little sensitivity to most of the Cl<sup>-</sup> channel blockers listed in Table III. The most frequently reported selectivity sequence for halogens is Br<sup>-</sup> > Cl<sup>-</sup> > I<sup>-</sup>, but the sequence varies from tissue to tissue, and I<sup>-</sup> > Br<sup>-</sup> > Cl<sup>-</sup> has also been reported. The lack of sensitivity of the NGB channel to glibenclamide is a clear difference with CFTR. Low-conductance Cl<sup>-</sup> channels have also been reported in cells that apparently do not express CFTR (Gabriel, Price, Boucher, and Stutts, 1992).

*Role of the Apical Membrane 10-pS Channel in Necturus Gallbladder Epithelium*

In secretory epithelia, apical membrane Cl<sup>-</sup> channels are a major pathway for transcellular electrogenic Cl<sup>-</sup> secretion, which generates a transepithelial voltage, in turn driving paracellular Na<sup>+</sup> secretion (Silva, Stoff, Field, Fine, Forrest, and Epstein, 1977; Greger and Schlatter, 1984). The transepithelial Na<sup>+</sup> flux is necessary to produce the net transepithelial solute flux responsible for water transport (Frizzell, Halm, Reckemmer, and Shoemaker, 1986; Frizzell and Halm, 1990; Anderson et al., 1992). Apical Cl<sup>-</sup> channels are the main sites for regulation of secretion, by way of phosphorylation catalyzed by PKA (Welsh, Li, McCann, Clancy, and Anderson, 1989). It is likely that the channel responsible for the process described above is CFTR, although channels other than CFTR have been demonstrated in secretory epithelia.

The functional role of apical membrane Cl<sup>-</sup> channels in absorptive epithelia is not obvious. Our studies indicate that these channels are not functional under basal conditions and that their activation is associated with inhibition of fluid absorption. Sustained Cl<sup>-</sup> secretion does not occur because of the lack of an appropriate Cl<sup>-</sup> uptake mechanism (e.g., Na-K-2Cl cotransport) at the basolateral membrane (Reuss, 1983). The decrease in fluid absorption is partially attributable to a fall in intracellular Cl<sup>-</sup> activity (i.e., Cl<sup>-</sup> entering the cell via Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange is lost to the apical bathing solution via the Cl<sup>-</sup> channel; Petersen and Reuss, 1983; Reuss, 1987). In addition to Cl<sup>-</sup> loss, activation of the Cl<sup>-</sup> channel causes apical membrane depolarization, which activates maxi-K<sup>+</sup> channels (Segal and Reuss, 1990a) and increases the driving force favoring electrodiffusive loss of K<sup>+</sup>. Finally, the net loss of KCl produces cell shrinkage (Cotton and Reuss, 1991). We speculate that the decrease in cell volume inhibits net salt transport across the basolateral membrane. These results support the idea of a reduction in basolateral membrane  $G_K$  upon cell shrinkage ( $R_b$  data in cAMP-stimulated tissues in the presence and absence of Cl<sup>-</sup> in the apical bathing solution; see Table I). However, the effect of cell volume changes on the rate of the Na<sup>+</sup> pump, shown in other systems for the case of hyposmotic cell swelling (Venosa, 1991), remains to be proven.

We thank Dr. G. A. Altenberg and Dr. S. A. Weinman for comments on a preliminary version of this paper, Dr. D. G. Brunder for computer-related support, B. F. Miller for technical assistance, and L. Durant for secretarial help.

This work was supported by NIH grant DK-38784.

*Original version received 11 February 1993 and accepted version received 30 April 1993.*

#### REFERENCES

- Altenberg, G., J. Copello, C. Cotton, K. Dawson, Y. Segal, F. Wehner, and L. Reuss. 1990. Electrophysiological methods for studying ion and water transport in *Necturus* gallbladder epithelium. *Methods in Enzymology*. 192:650–683.
- Anderson, M. P., D. N. Sheppard, H. A. Berger, and M. J. Welsh. 1992. Chloride channels in the apical membrane of normal and cystic fibrosis airway and intestinal epithelia. *American Journal of Physiology*. 263:L1–L14.
- Cabantchik, Z. I., and R. Greger. 1992. Chemical probes for anion transporters of mammalian cell membranes. *American Journal of Physiology*. 262:C803–C826.
- Colquhoun, D., and A. G. Hawkes. 1983. The principles of the stochastic interpretation of ion-channel mechanisms. In *Single Channel Recording*. E. Neher and B. Sakmann, editors. Plenum Publishing Corp., New York. 135–175.
- Copello, J., F. Wehner, and L. Reuss. 1993. Artificial expression of maxi-K<sup>+</sup> channels in basolateral membrane of gallbladder epithelial cells. *American Journal of Physiology (Cell)*. 264:C1128–C1136.
- Cotton, C. U., and L. Reuss. 1991. Effects of changes in mucosal solution Cl<sup>-</sup> or K<sup>+</sup> concentration on cell water volume of *Necturus* gallbladder epithelium. *Journal of General Physiology*. 97:667–686.
- Duffey, M. E., B. Hainau, S. Ho, and C. J. Bentzel. 1981. Regulation of epithelial tight junction permeability by cyclic AMP. *Nature*. 294:451–453.
- Eisenman, G. 1962. Cation selective glass electrodes and their mode of operation. *Biophysical Journal*. 2:S259–S323.

- Finn, A. L., M. Dillard, and M. Gaido. 1993. Independently-gated multiple substates of an epithelial chloride channel protein. *Proceedings of the National Academy of Sciences, USA*. In press.
- Finn, A. L., L.-M. Tsai, and R. J. Falk. 1989. Monoclonal antibodies to the apical chloride channel in *Necturus* gallbladder inhibit the chloride conductance. *Proceedings of the National Academy of Sciences, USA*. 86:7649–7652.
- Frizzell, R. A., and D. R. Halm. 1990. Chloride channels in epithelial cells. *Current Topics in Membranes and Transport*. 37:247–282.
- Frizzell, R. A., D. R. Halm, G. Rechkemmer, and R. L. Shoemaker. 1986. Chloride channel regulation in secretory epithelia. *Federation Proceedings*. 45:2727–2731.
- Frömter, E. 1972. The route of passive ion movement through the epithelium of *Necturus* gallbladder. *Journal of Membrane Biology*. 8:259–301.
- Fuller, C. M., and D. J. Benos. 1992. CFTR! *American Journal of Physiology*. 263:C267–C286.
- Gabriel, S. I., E. M. Price, R. C. Boucher, and M. J. Stutts. 1992. Small linear chloride channels are endogenous to nonepithelial cells. *American Journal of Physiology*. 263:C708–C713.
- Gögelein, H. 1988. Chloride channels in epithelia. *Biochimica et Biophysica Acta*. 947:521–574.
- Greger, R., and E. Schlatter. 1984. Mechanism of NaCl secretion in the rectal gland of spiny dogfish (*Squalus acanthias*). I. Experiments in isolated in vitro perfused rectal gland tubules. *Pflügers Archiv*. 402:63–75.
- Heming, T., J. Copello, and L. Reuss. 1992. Apical chloride channels in *Necturus* gallbladder (NGB) epithelium. *FASEB Journal*. 6:A535. (Abstr.)
- Hille, B. 1984. *Ion Channels in Excitable Membranes*. Sinauer Associates, Inc., Sunderland, MA. 426 pp.
- Kitchens, D. L., K. Dawson, and L. Reuss. 1990. Zinc blocks apical membrane anion exchange in gallbladder epithelium. *American Journal of Physiology*. 258:G745–G752.
- Krick, W., J. Disser, A. Hazama, G. Burckhardt, and E. Frömter. 1991. Evidence for a cytosolic inhibitor of epithelial chloride channels. *Pflügers Archiv*. 418:491–499.
- Kunzelmann, K., M. Tilmann, C. P. Hansen, and R. Greger. 1991. Inhibition of epithelial chloride channels by cytosol. *Pflügers Archiv*. 418:479–490.
- Lipkowitz, M. S., and R. G. Abramson. 1989. Differential permeabilities of rat renal brush border and basolateral membrane vesicles. *American Journal of Physiology*. 256:F18–F28.
- Marunaka, Y., and D. C. Eaton. 1990. Effect of insulin and phosphatase on a  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channel in a distal nephron cell line (A6). *Journal of General Physiology*. 95:773–789.
- McCann, J. D., and M. J. Welsh. 1990. Regulation of  $\text{Cl}^-$  and  $\text{K}^+$  channels in airway epithelium. *Annual Review of Physiology*. 52:115–135.
- Petersen, K.-U., and L. Reuss. 1983. Cyclic AMP-induced chloride permeability in the apical membrane of *Necturus* gallbladder epithelium. *Journal of General Physiology*. 81:705–729.
- Reeves, W. B., and T. E. Andreoli. 1992. Renal epithelial chloride channels. *Annual Review of Physiology*. 54:29–50.
- Reuss, L. 1983. Basolateral KCl co-transport in a NaCl-absorbing epithelium. *Nature*. 305:723–726.
- Reuss, L. 1987. Cyclic AMP inhibits  $\text{Cl}^-/\text{HCO}_3^-$  exchange at the apical membrane of *Necturus* gallbladder epithelium. *Journal of General Physiology*. 90:173–196.
- Reuss, L. 1989. Ion transport across gallbladder epithelium. *Physiological Reviews*. 69:503–545.
- Reuss, L., and J. L. Costantin. 1984.  $\text{Cl}^-/\text{HCO}_3^-$  exchange at the apical membrane of *Necturus* gallbladder. *Journal of General Physiology*. 83:801–818.
- Reuss, L., J. L. Costantin, and J. E. Bazile. 1987. Diphenylamine-2-carboxylate blocks  $\text{Cl}^-/\text{HCO}_3^-$  exchange in *Necturus* gallbladder epithelium. *American Journal of Physiology*. 253:C79–C89.

- Reuss, L., and A. L. Finn. 1975a. Electrical properties of the cellular transepithelial pathway in *Necturus* gallbladder. I. Circuit analysis and steady-state effects of mucosal solution ionic substitutions. *Journal of Membrane Biology*. 25:115–139.
- Reuss, L., and A. L. Finn. 1975b. Electrical properties of the cellular transepithelial pathway in *Necturus* gallbladder. II. Ionic permeability of the apical cell membrane. *Journal of Membrane Biology*. 25:141–161.
- Reuss, L., and K.-U. Petersen. 1985. Cyclic AMP inhibits Na<sup>+</sup>/H<sup>+</sup> exchange at the apical membrane of *Necturus* gallbladder epithelium. *Journal of General Physiology*. 85:409–429.
- Reuss, L., Y. Segal, and G. Altenberg. 1991. Regulation of ion transport across gallbladder epithelium. *Annual Review of Physiology*. 53:361–373.
- Segal, Y., and L. Reuss. 1989. Cl<sup>-</sup> channels in cyclic AMP-stimulated gallbladder epithelium. *FASEB Journal*. 3:A862. (Abstr.)
- Segal, Y., and L. Reuss. 1990a. Maxi K<sup>+</sup> channels and their relationship to the apical membrane conductance in *Necturus* gallbladder epithelium. *Journal of General Physiology*. 95:791–818.
- Segal, Y., and L. Reuss. 1990b. Ba<sup>2+</sup>, TEA<sup>+</sup>, and quinine effects on apical membrane K<sup>+</sup> conductance and maxi K<sup>+</sup> channels in gallbladder epithelium. *American Journal of Physiology*. 259:C56–C68.
- Sheppard, D. N., and M. J. Welsh. 1992. Effect of ATP-sensitive K<sup>+</sup> channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. *Journal of General Physiology*. 100:573–591.
- Silva, P., J. Stoff, M. Field, L. Fine, J. N. Forrest, and F. H. Epstein. 1977. Mechanism of active chloride secretion by shark rectal gland: role of Na-K-ATPase in chloride transport. *American Journal of Physiology*. 233:F298–F306.
- Stewart, C. P., J. M. Winterhager, K. Heintze, and K.-U. Petersen. 1989. Electrogenic bicarbonate secretion by guinea pig gallbladder epithelium: apical membrane exit. *American Journal of Physiology*. 256:C736–C749.
- Stoddard, J. S., and L. Reuss. 1988. Voltage- and time dependence of apical membrane conductance during current clamp in *Necturus* gallbladder epithelium. *Journal of Membrane Biology*. 103:191–204.
- Stoddard, J. S., and L. Reuss. 1989. Electrophysiologic effects of mucosal Cl<sup>-</sup> removal in *Necturus* gallbladder epithelium. *American Journal of Physiology*. 257:C568–C578.
- Tsai, L.-M., M. Dillard, R. L. Rosenberg, R. J. Falk, M. L. Gaido, and A. L. Finn. 1991. Reconstitution of an epithelial chloride channel. Conservation of the channel from mudpuppy to man. *Journal of General Physiology*. 98:723–750.
- Valverde, M. A., M. Díaz, F. V. Sepúlveda, D. R. Gill, S. C. Hyde, and C. F. Higgins. 1992. Volume-regulated chloride channels associated with the human multidrug-resistance P-glycoprotein. *Nature*. 355:830–833.
- Venosa, R. A. 1991. Hypo-osmotic stimulation of active Na<sup>+</sup> transport in frog muscle: apparent upregulation of Na<sup>+</sup> pumps. *Journal of Membrane Biology*. 120:97–104.
- Welsh, M. J., M. P. Anderson, D. P. Rich, H. A. Berger, G. M. Denning, L. S. Ostedgaard, D. N. Sheppard, S. H. Cheng, R. J. Gregory, and A. E. Smith. 1992. Cystic fibrosis transmembrane conductance regulator: a chloride channel with novel regulation. *Neuron*. 8:821–829.
- Welsh, M. J., M. Li, J. D. McCann, J. P. Clancy, and M. P. Anderson. 1989. Phosphorylation-dependent regulation of apical membrane chloride channels in normal and cystic fibrosis airway epithelium. *Annals of the New York Academy of Sciences*. 574:44–51.
- Wright, E. M., and J. M. Diamond. 1977. Anion selectivity in biological systems. *Physiological Reviews*. 57:109–156.