

# Regulation of cAMP-Activated Apical Membrane Chloride Conductance in Gallbladder Epithelium

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**ABSTRACT** Regulation of the cAMP-activated apical membrane  $\text{Cl}^-$  conductance ( $G_{\text{Cl}}^a$ ) in *Necturus* gallbladder (NGB) epithelial cells was investigated with intracellular-microelectrode techniques.  $G_{\text{Cl}}^a$  was increased by exposure to 8-Br-cAMP, theophylline or forskolin. Neither 8-Br-cGMP nor elevation of intracellular  $[\text{Ca}^{2+}]$  using ionomycin had effects on  $G_{\text{Cl}}^a$  or interfered with activation of  $G_{\text{Cl}}^a$  by forskolin. *N*-(2-[methylamino]ethyl)-5-isoquinolinesulfonamide (H8), an inhibitor of cAMP-dependent protein kinase (PKA), slowed but did not prevent the  $G_{\text{Cl}}^a$  response to 8-Br-cAMP. Phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C (PKC), stimulated  $G_{\text{Cl}}^a$  but had no effects on intracellular [cAMP].  $G_{\text{Cl}}^a$  was unaffected by 4 $\alpha$ -phorbol, a PMA analog which does not activate PKC. Okadaic acid (OA), an inhibitor of protein phosphatases (PP) types 1 and 2A, slowed the activation of  $G_{\text{Cl}}^a$  by 8-Br-cAMP, hastened the return of  $G_{\text{Cl}}^a$  to basal values following removal of 8-Br-cAMP, and significantly reduced the elevation in intracellular [cAMP] produced by forskolin. OA had no effects on the  $G_{\text{Cl}}^a$  changes elicited by theophylline. We conclude that: (a) NGB  $G_{\text{Cl}}^a$  can be activated by PKA-mediated phosphorylation of apical membrane  $\text{Cl}^-$  channels or a regulatory protein, (b)  $G_{\text{Cl}}^a$  can also be activated via PKC, by a cAMP-independent mechanism, (c) OA-sensitive PP are not required for inactivation of  $G_{\text{Cl}}^a$ ; OA appears to stimulate phosphodiesterase, which lowers intracellular [cAMP] and affects  $G_{\text{Cl}}^a$  activation, and (d) the apical membrane of NGB epithelium lacks a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance.

## INTRODUCTION

The apical membrane of *Necturus* gallbladder (NGB) epithelial cells is primarily  $\text{K}^+$  selective and lacks a significant  $\text{Cl}^-$  conductance ( $G_{\text{Cl}}^a$ ) under basal conditions (Reuss, 1989). However, a large  $G_{\text{Cl}}^a$  is elicited by agents that elevate the intracellular [cAMP] ( $[\text{cAMP}]_i$ ) (Petersen and Reuss, 1983). Activation of  $G_{\text{Cl}}^a$  results in loss of intracellular  $\text{Cl}^-$  and depolarization of the apical membrane; the latter activates apical membrane maxi- $\text{K}^+$  channels and increases the driving force for  $\text{K}^+$  efflux from the cells (Cotton and Reuss, 1991). The consequences are that (a)  $\text{Cl}^-$  influx via the apical membrane

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$\text{Cl}^-/\text{HCO}_3^-$  exchanger is "short-circuited" by efflux via the  $\text{Cl}^-$  conductive pathway, (b) the cells shrink because of net loss of KCl (and water) across the apical membrane, and (c) the rate of transepithelial fluid absorption is reduced (Petersen and Reuss, 1983; Reuss, 1987; Cotton and Reuss, 1991).

The channel responsible for the cAMP-activated  $G_{\text{Cl}}^a$  in NGB epithelium has the following characteristics: (a) conductance of ca. 10 pS, (b) linear current-voltage relationship, (c) voltage-,  $\text{Ca}^{2+}$ - and pH-insensitivity, (d) anion-selectivity sequence of  $\text{SCN}^- > \text{NO}_3^- > \text{I}^- > \text{Br}^- > \text{Cl}^-$ , and (e) insensitivity to many agents reported to block  $\text{Cl}^-$  channels in other cells (Copello, Heming, Segal, and Reuss, 1993). Many of these characteristics resemble those of the human cystic fibrosis transmembrane conductance regulator (CFTR)  $\text{Cl}^-$  channel (reviewed by Anderson, Sheppard, Berger, and Welsh, 1992; Fuller and Benos, 1992; Hanrahan, Tabcharani, and Grygorczyk, 1993). Hence, an important question is whether regulation of the NGB channel is also similar to that of CFTR.

Activation of  $G_{\text{Cl}}^a$  in NGB epithelium could reflect (a) phosphorylation and activation of "silent" channels present in the apical membrane (e.g., CFTR  $\text{Cl}^-$  channels: Berger, Travis, and Welsh, 1993), (b) insertion of channels from a subcellular vesicular pool (e.g.,  $\text{Cl}^-$  channels of HT<sub>29</sub> cells: Allert, Leipziger, and Greger, 1992), or (c) a combination of the two processes. There are strong arguments for involvement of protein kinase A (PKA) in activation of NGB  $G_{\text{Cl}}^a$ . First,  $G_{\text{Cl}}^a$  in intact tissues is activated by 8-Br-cAMP (a permeant analog of cAMP), by forskolin which stimulates cAMP production through activation of adenylate cyclase (AC), and by theophylline which reduces the rate of cAMP degradation through inhibition of phosphodiesterase (PDE) (Petersen and Reuss, 1983; Reuss and Petersen, 1985). Second, basal  $G_{\text{Cl}}^a$  (when present) can be blocked by a cAMP analog that inhibits PKA and is not degraded by PDE (Garvin and Spring, 1992). Third, the 10-pS  $\text{Cl}^-$  channel in excised apical membrane patches can be activated with PKA plus ATP from the cytosolic surface (Copello et al., 1993).

Other kinases could also play modulatory roles. For example, protein kinase C (PKC) activates low-conductance  $\text{Cl}^-$  channels in CFTR-transfected CHO cells (Tabcharani, Chang, Riordan, and Hanrahan, 1991), whereas cGMP-dependent protein kinase (cGK) activates similar channels in T84 cells (Lin, Nairn, and Guggino, 1992). Furthermore, CFTR expressed in fibroblasts is phosphorylated by PKA, PKC, and cGK, although only PKA and PKC activate a plasma membrane  $\text{Cl}^-$  conductance ( $G_{\text{Cl}}$ ) (Berger et al., 1993).

Cyclic-AMP-mediated activation of NGB  $G_{\text{Cl}}^a$  is fully reversible (Petersen and Reuss, 1983), which suggests a role for dephosphorylation presumably catalyzed by protein phosphatases (PP). Okadaic acid (OA), an inhibitor of PP types 1 and 2A (Ishihara, Martin, Brautigan, Karaki, Ozaki, Kato, Fusetani, Watabe, Hashimoto, Uemura, and Hartshorne, 1989), activates  $G_{\text{Cl}}^a$  in epithelial cells from shark rectal gland (La, Carosi, Valentich, Shenolikar, and Sansom, 1991). In cardiomyocytes, OA potentiates the stimulation of  $G_{\text{Cl}}$  by isoproterenol and forskolin, and prevents the fall in  $G_{\text{Cl}}$  after agonist removal (Hwang, Horie, and Gadsby, 1993). In NGB epithelium, OA has been reported to reverse the block of  $G_{\text{Cl}}^a$  produced by a monoclonal antibody raised against NGB epithelial cells (Finn, Gaido, Dillard, and Brautigan, 1992).

The principal aim of this study was to investigate the mechanisms of regulation of

the cAMP-activated G<sub>Cl</sub><sup>a</sup> of NGB epithelium. The results indicate that G<sub>Cl</sub><sup>a</sup> is activated by PKA-dependent phosphorylation of the channel itself or a regulatory protein. PKC also activates the conductive pathway, independently of the cAMP-mediated mechanism. G<sub>Cl</sub><sup>a</sup> was not activated by 8-Br-cGMP or by elevation of intracellular [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>i</sub>) using ionomycin. Moreover, neither 8-Br-cGMP nor ionomycin interfered with cAMP-mediated activation of G<sub>Cl</sub><sup>a</sup>. While OA by itself did not activate G<sub>Cl</sub><sup>a</sup>, it did alter the response of NGB to 8-Br-cAMP, most likely via stimulation of PDE.

Portions of this work have previously appeared in abstract form (Heming, Copello, and Reuss, 1993).

## MATERIALS AND METHODS

### General Methods

Mudpuppies (*Necturus maculosus*) were obtained from NASCO (Ft. Atkinson, WI) or Kons Scientific Co., Inc. (Germantown, WI), kept in tap water at 5–10°C, and anesthetized by immersion in a 1 g/l solution of tricaine methanesulfonate. Mudpuppies were killed by intracardiac KCl injection. The gallbladders were excised, sliced open, and mounted apical side-up in a modified Ussing chamber at room temperature (23 ± 1°C), as previously described (Altenberg, Copello, Cotton, Dawson, Segal, Wehner, and Reuss, 1990). The control bathing solution, hereafter referred to as physiologic salt solution (PSS), contained (in mM) 90 NaCl, 10 NaHCO<sub>3</sub>, 2.5 KCl, 1.8 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, and 0.5 Na-phosphate. PSS was equilibrated with a gas mixture of 1% CO<sub>2</sub>-99% air and had a pH of ~7.65 at room temperature. In some experiments, Cl<sup>-</sup> was partially replaced with cyclamate at constant [Na<sup>+</sup>], or Na<sup>+</sup> was partially replaced with K<sup>+</sup>, Ba<sup>2+</sup>, tetramethylammonium (TMA<sup>+</sup>), or tetraethylammonium (TEA<sup>+</sup>) at constant [Cl<sup>-</sup>]. All ionic substitutions were isomolar.

G<sub>Cl</sub><sup>a</sup> was activated by treating tissues with either 8-Br-cAMP (0.25–1.0 mM, apical side), theophylline (3 mM, basolateral side), or forskolin (2.5 μM, apical or basolateral side). Tissues treated with these agents are referred to as “stimulated.” Additional treatments were 8-Br-cGMP (1 mM, apical or basolateral side), phorbol 12-myristate 13-acetate (PMA) or 4α-phorbol (100 nM, apical side), ionomycin (10 μM, apical and basolateral sides), *N*-[2-(methylamino)-ethyl]-5-isoquinoline-sulfonamide (H8) (10 μM, apical side), and OA (0.4–20 μM, apical side).

### Electrophysiological Techniques

Transepithelial voltage ( $V_{ms}$ ), transepithelial resistance ( $R_t$ ), cell membrane voltages (apical,  $V_{mc}$ ; basolateral,  $V_{cs}$ ), and the apparent ratio of apical and basolateral membrane resistances ( $R_a/R_b$ ; subscripts *a* and *b* denote apical and basolateral membranes, respectively) were measured as previously described (Altenberg et al., 1990). Single-barrel microelectrodes were prepared from borosilicate glass (1 mm OD, 0.5 mm ID) 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 MΩ when immersed in PSS. The reference electrode in the basolateral bathing compartment was a Ag-AgCl pellet in series with a PSS-agar bridge. The electrode in the apical bathing compartment was a flowing saturated KCl junction or a PSS-agar bridge, either in series with a calomel half-cell. The flowing junction was used in studies involving ionic substitutions because it minimizes changes in liquid-junction potentials. The PSS-agar bridge was used in all other studies. Transepithelial current pulses of 50 μA cm<sup>-2</sup>, 2-s duration, and frequency of 2–3 min<sup>-1</sup> were applied across the tissue through Ag-AgCl electrodes.  $R_t$  and  $R_a/R_b$  were determined from the resulting voltage deflections (corrected for series resistances) at 600 ms after the onset of each pulse.

### *Measurements of [cAMP]<sub>i</sub>*

Gallbladders were divided in two pieces (one experimental, one control), mounted apical side-up on Sylgard-coated dishes, and incubated in PSS at room temperature with or without one or more of the following agents: forskolin, OA, H8, PMA and *N,N*-dimethylformamide (DMF) (see Results for concentrations and incubation times). The epithelial cell layer was rapidly removed by scraping with a blade, aspirated, mixed with ethanol (equal volumes of ethanol and PSS-suspended cells), and centrifuged (20 min, 1,000 rpm, 4°C). The supernatant was used to measure cAMP with a commercial radioimmunoassay kit (Amersham's Cyclic-AMP [<sup>3</sup>H] assay system TRK 432, Amersham Corp., Arlington Heights, IL). The pellet was treated with 0.1 M NaOH (1 ml, 12 h, 4°C) to dissolve alkali-soluble protein; protein content of the extract was determined using the Coomassie plus protein assay reagent (Pierce Chemical Co., Rockford, IL).

### *Chemicals*

H8 was purchased from Research Biochemicals Inc. (Natick, MA), ionomycin from Calbiochem Corp. (La Jolla, CA), and OA from Moana Bioproducts Inc. (Honolulu, HA). All other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

PMA was dissolved in dimethyl sulfoxide (DMSO) and OA was dissolved in DMF. DMSO has no effects on the bioelectrical properties of NGB epithelium (Altenberg, Subramanyam, Bergmann, Johnson, and Reuss, 1993). DMF alone depolarized the cell membranes slightly and reduced  $R_a/R_b$  in two of three experiments, but had no effects on  $G_{Cl}^a$  (data not shown). These effects of DMF were fully and rapidly reversible. Effects of OA are virtually irreversible (Jennings and Schulz, 1991). Hence, studies of the effects of OA were usually performed after removal of both OA and DMF from the bathing solutions.

### *Statistics*

The data are presented as means  $\pm$  SEM. Statistical comparisons were made using two-tailed *t*-tests for paired or unpaired samples, as appropriate. A value of  $P < 0.05$  was considered significant.

## RESULTS

### *Effects of Elevating [cAMP]<sub>i</sub>*

Elevation of [cAMP]<sub>i</sub> using 8-Br-cAMP, theophylline or forskolin produced a slow depolarization of both cell membranes and a large decrease in  $R_a/R_b$  (Table I). Fig. 1 illustrates the effects of lowering apical solution [Cl<sup>-</sup>] before and after forskolin. In control (unstimulated) tissues, reducing apical solution [Cl<sup>-</sup>] to 8 mM caused a slow hyperpolarization of both cell membranes with little change in  $R_a/R_b$ , denoting the lack of a significant basal  $G_{Cl}^a$ . In tissues stimulated with forskolin, reduction of apical solution [Cl<sup>-</sup>] produced a rapid transient depolarization of both cell membranes followed by a slow repolarization. Restoration of apical solution [Cl<sup>-</sup>] caused mirror-image effects, namely a rapid hyperpolarization of  $V_{mc}$  and  $V_{cs}$ . These effects are consistent with the presence of a large  $G_{Cl}^a$ . Similar results were obtained when tissues were stimulated with 8-Br-cAMP and theophylline (data not shown). In two of seven experiments, tissues did not respond to theophylline but did respond to subsequent concurrent addition of 8-Br-cAMP, suggesting that the rate of endogenous cAMP production is variable. The effects of 8-Br-cAMP, theophylline, and forskolin were fully reversible after removal of the agonist (data not shown). These

TABLE I  
Effects of 8-Br-cAMP, Theophylline, and Forskolin on Voltages and Resistances

Condition	<i>n</i>	V <sub>ms</sub> mV	V <sub>mc</sub> mV	R <sub>a</sub> /R <sub>b</sub>	R <sub>t</sub> Ω cm <sup>2</sup>
Control	11	0.0 ± 0.3	-76 ± 2	6.23 ± 1.59	161 ± 18
8-Br-cAMP	11	-1.2 ± 0.4*	-56 ± 3*	0.09 ± 0.04*	201 ± 24*
Control	5	0.1 ± 0.6	-72 ± 2	5.05 ± 1.98	219 ± 37
Theophylline	5	-1.3 ± 0.6*	-46 ± 1*	0.04 ± 0.02*	235 ± 36
Control	5	-0.4 ± 0.1	-77 ± 4	6.47 ± 1.08	218 ± 24
Forskolin	5	-1.9 ± 0.5*	-58 ± 2*	0.08 ± 0.01*	246 ± 23*

Values are means ± SEM (*n* = number of tissues). Treatments were 1 mM 8-Br-cAMP (apical side), 3 mM theophylline (basolateral side), and 2.5 μM forskolin (basolateral side). Data are apparent steady state values. V<sub>ms</sub> and V<sub>mc</sub> denote transepithelial and apical membrane voltages, respectively (V<sub>ms</sub> reference = basolateral bathing solution; V<sub>mc</sub> reference = apical bathing solution). R<sub>a</sub>/R<sub>b</sub>, apparent ratio of cell membrane resistances; R<sub>t</sub>, transepithelial resistance. \*Significantly different from paired control value.

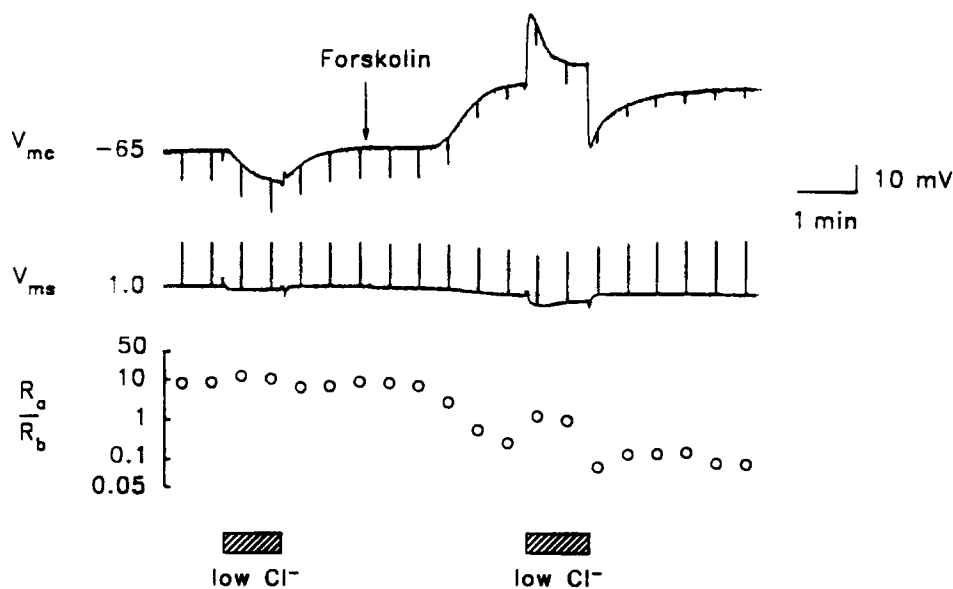


FIGURE 1. Effects of transient (1 min) reduction of apical solution [Cl<sup>-</sup>] to 8 mM (low Cl<sup>-</sup>) by isomolar replacement with cyclamate, under control conditions and after exposure to forskolin (2.5 μM, apical side). V<sub>ms</sub> and V<sub>mc</sub> denote transepithelial and apical membrane voltages. Values indicate voltages at beginning of records (V<sub>ms</sub> reference = basolateral bathing solution; V<sub>mc</sub> reference = apical bathing solution). Voltage deflections were caused by transepithelial current pulses of 50 μA · cm<sup>-2</sup>; the transepithelial resistance (R<sub>t</sub>) and the apparent ratio of cell membrane resistances (R<sub>a</sub>/R<sub>b</sub>) were calculated from these deflections after correction for series resistances. Before forskolin, Cl<sup>-</sup> replacement produced a slow hyperpolarization of V<sub>mc</sub>. Exposure to forskolin caused V<sub>mc</sub> to depolarize by ca. 20 mV and R<sub>a</sub>/R<sub>b</sub> to fall almost to zero. Cl<sup>-</sup> replacement after forskolin caused a rapid depolarization of V<sub>mc</sub> followed by partial repolarization and an increase in R<sub>a</sub>/R<sub>b</sub> (see text and Table I). Restoration of apical solution [Cl<sup>-</sup>] had mirror-image effects. These responses are characteristic of a large apical membrane electrodiffusive P<sub>Cl</sub>. Forskolin effects were fully reversible after agonist removal (data not shown).

responses have been described in detail previously (Petersen and Reuss, 1983; Reuss and Petersen, 1985; Reuss, 1987; Stoddard and Reuss, 1989; Cotton and Reuss, 1991).

*Effects of Elevating Intracellular [cGMP]*

As illustrated in Fig. 2, 8-Br-cGMP (1 mM, basolateral side), a permeant analog of cGMP, had no effects on basal membrane voltages or resistances, on the responses to a reduction in apical solution  $[Cl^-]$ , or on the responses elicited by forskolin. In the

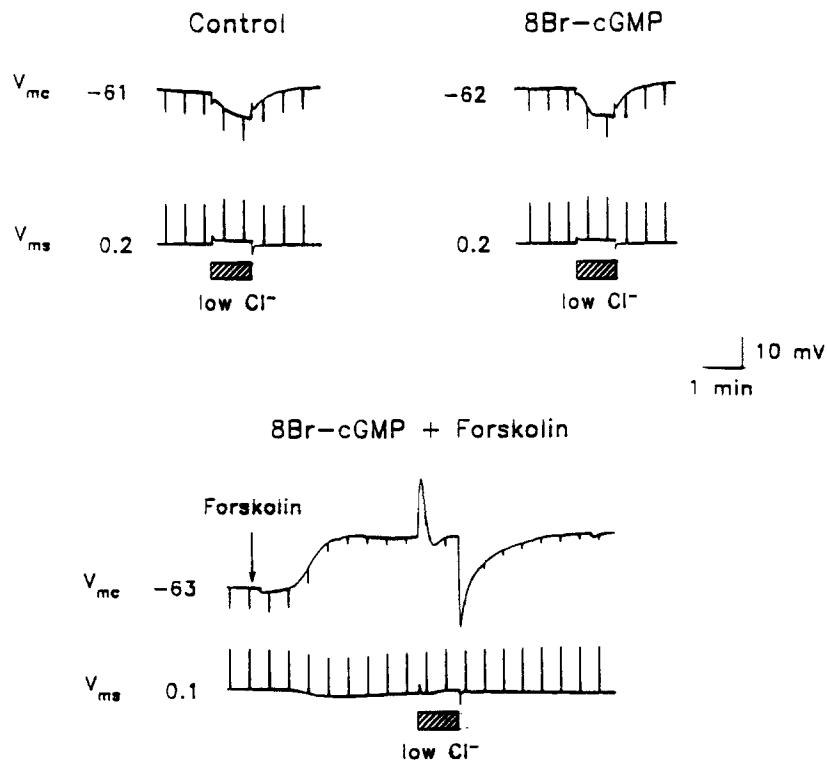


FIGURE 2. Effects of 8-Br-cGMP (1 mM, basolateral side) on voltages and resistances, and on changes elicited by reducing apical solution  $[Cl^-]$ . The records are from a single tissue and are typical of results from six experiments. See Fig. 1 for abbreviations. (Top) Basal voltages and resistances were not altered by 20-min exposure to 8-Br-cGMP. Likewise, the responses to a transient reduction in apical solution  $[Cl^-]$  were unchanged. Thus, cGMP did not activate  $G_{Cl}^a$ . (Bottom) 8-Br-cGMP did not interfere with activation of  $G_{Cl}^a$  by forskolin.

presence of 8-Br-cGMP, forskolin (2.5  $\mu$ M, apical side) caused a  $\sim 20$  mV depolarization of  $V_{mc}$  and a decrease in  $R_a/R_b$  from  $5.64 \pm 0.94$  to  $0.07 \pm 0.02$  ( $n = 6$ ). Reduction of apical solution  $[Cl^-]$  in the presence of 8-Br-cGMP and forskolin caused a rapid transient  $V_{mc}$  depolarization of  $27 \pm 3$  mV ( $n = 5$ ). These results were not different from those obtained with forskolin alone (cf Table I and Fig. 1). Similar results were obtained when 1 mM 8-Br-cGMP was added to the apical bathing

solution ( $n = 5$ ) (data not shown). In conclusion, elevation of  $[cGMP]_i$  does not activate  $G_{Cl}^a$  or interfere with forskolin-mediated activation of  $G_{Cl}^a$ .

#### Effects of Elevating $[Ca^{2+}]_i$

To test for a  $Ca^{2+}$ -activated  $G_{Cl}^a$ , tissues were treated with ionomycin which is known to elevate  $[Ca^{2+}]_i$  in NGB epithelium (Altenberg, Stoddard, and Reuss, 1992). TEA<sup>+</sup> (4.5 mM) was added to the apical bathing solution to block  $Ca^{2+}$ -activated maxi-K<sup>+</sup> channels. Ionomycin alone (10  $\mu$ M, apical and basolateral sides) produced a  $\sim 13$  mV

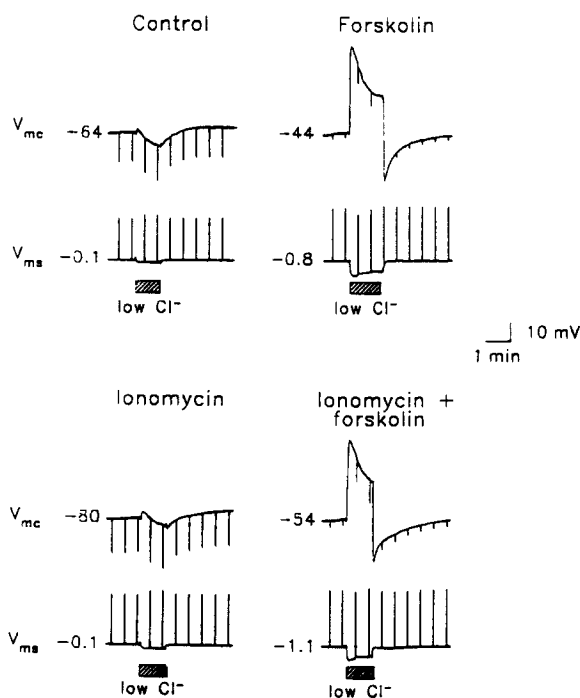


FIGURE 3. Effects of ionomycin (10  $\mu$ M, apical and basolateral sides, apical bathing solution containing 4.5 mM TEA<sup>+</sup>) on voltages and resistances, and on changes elicited by reducing apical solution  $[Cl^-]$ . The records are from a single tissue; similar results were obtained in seven experiments. See Fig. 1 for abbreviations. (Control) Partial  $Cl^-$  replacement by cyclamate (low  $Cl^-$ ) produced a slow hyperpolarization of  $V_{mc}$  consistent with the absence of a detectable  $G_{Cl}^a$ . (Forskolin) After 10 min in forskolin (2.5  $\mu$ M, apical side),  $V_{mc}$  depolarized by  $\sim 20$  mV and  $R_a/R_b$  fell almost to zero. The response to reducing apical solution  $[Cl^-]$  is indicative of a large  $G_{Cl}^a$ . (Ionomycin) Elevation of  $[Ca^{2+}]_i$  by exposure to iono-

mycin caused a hyperpolarization of  $V_{mc}$  but did not greatly affect  $R_a/R_b$ . Partial  $Cl^-$  replacement produced similar results to those observed under control conditions. (Ionomycin + forskolin) Reducing apical solution  $[Cl^-]$  had the same effects as with forskolin alone. These data indicate that NGB epithelium lacks a  $Ca^{2+}$ -activated  $G_{Cl}^a$ , and that increases in  $[Ca^{2+}]_i$  do not interfere with forskolin-mediated activation of  $G_{Cl}^a$ .

hyperpolarization of  $V_{mc}$  and a decrement in  $R_a/R_b$  from  $11.33 \pm 2.49$  to  $7.12 \pm 1.26$  ( $n = 7$ ), probably due to incomplete block of apical membrane maxi-K<sup>+</sup> channels.  $V_{ms}$  and  $R_t$  did not change significantly (data not shown). Replacement of apical solution  $Cl^-$  with cyclamate had similar effects before and after ionomycin (Fig. 3). Ionomycin also had no effect on the responses elicited by forskolin. Forskolin (2.5  $\mu$ M, apical side) produced a  $\sim 20$ – $25$  mV depolarization of  $V_{mc}$  and caused  $R_a/R_b$  to fall to 0.05–0.06 in either the presence or absence of ionomycin. Ionomycin did not alter the time course of the responses to forskolin (data not shown). Further,

replacing apical solution  $\text{Cl}^-$  with cyclamate produced similar depolarizations of  $V_{\text{mc}}$  with and without ionomycin ( $41 \pm 1$  and  $40 \pm 2$  mV, respectively). These  $V_{\text{mc}}$  depolarizations are larger than those observed under other conditions (e.g., 8-Br-cGMP plus forskolin) because the TEA<sup>+</sup>-block of apical membrane  $\text{K}^+$  conductance increases the apical membrane transference number for  $\text{Cl}^-$ . These data indicate that NGB epithelium lacks a  $\text{Ca}^{2+}$ -activated  $G_{\text{Cl}}^{\text{a}}$  and that increases in  $[\text{Ca}^{2+}]_{\text{i}}$  do not interfere with forskolin-mediated activation of  $G_{\text{Cl}}^{\text{a}}$ .

#### *Effects of PKA Inhibition*

Pretreatment with H8 (10  $\mu\text{M}$ , 60 min, apical side), a competitive inhibitor of PKA (Hidaka, Inagaki, Kawamoto, and Sasaki, 1984), had no effects on the voltages or resistances of control tissues (Table II). Likewise, H8 did not significantly alter the steady state responses to 8-Br-cAMP. Exposure to 1 mM 8-Br-cAMP (apical side) produced a  $\sim 20$  mV depolarization of  $V_{\text{mc}}$  and caused  $R_{\text{a}}/R_{\text{b}}$  to fall to 0.06–0.09, either before or after H8 (Table II). However, H8 slowed the  $R_{\text{a}}/R_{\text{b}}$  responses elicited by 8-Br-cAMP (Fig. 4). To assess whether these effects could be due to a decrease in  $[\text{cAMP}]_{\text{i}}$ , in separate studies we measured the  $[\text{cAMP}]_{\text{i}}$  in tissues exposed to 2.5  $\mu\text{M}$

TABLE II  
*Effects of H8 on Voltages and Resistances, in Absence and Presence of 8-Br-cAMP*

Condition	[8-Br-cAMP]	[H8]	$V_{\text{mc}}$	$R_{\text{a}}/R_{\text{b}}$
	mM	$\mu\text{M}$	mV	
A	0	0	$-76 \pm 2$	$8.87 \pm 3.44$
B	0	10	$-82 \pm 2$	$9.92 \pm 6.24$
C	1	0	$-56 \pm 4^*$	$0.09 \pm 0.04^*$
D	1	10	$-60 \pm 2^*$	$0.06 \pm 0.02^*$

Values are means  $\pm$  SEM ( $n = 6$ ). Data are apparent steady state values. See Table I for abbreviations. \*Significant effect elicited by 8-Br-cAMP (C vs A or D vs B).

forskolin in the presence and absence of H8. H8 had no effect on  $[\text{cAMP}]_{\text{i}}$  at 8 min of exposure to forskolin ( $60 \pm 18$  and  $119 \pm 30$  pmol/mg protein, control and H8, respectively,  $n = 5$ ) and significantly increased, instead of decreasing,  $[\text{cAMP}]_{\text{i}}$  at 16 min ( $173 \pm 67$  and  $401 \pm 110$  pmol/mg protein, control and H8, respectively,  $n = 5$ ). Thus, H8 slowed the responses to 8-Br-cAMP without affecting the magnitude of the maximal response and without decreasing  $[\text{cAMP}]_{\text{i}}$ .

#### *Effects of PKC Activation*

To assess PKC effects, tissues were treated with phorbol esters (Evans, Parker, Olivier, Thomas, Ryves, Evans, Gordge, and Sharma, 1991). As illustrated in Fig. 5 and summarized in Table III,  $G_{\text{Cl}}^{\text{a}}$  was activated by PMA (100 nM, apical side). The steady state changes in voltages and resistances induced by PMA were comparable to those produced by elevation of  $[\text{cAMP}]_{\text{i}}$  (cf. Table I). Although the exposure to PMA lasted 3–5 min, its effects persisted for at least 90 min.  $4\alpha$ -phorbol, a PMA analog which does not activate PKC (Evans et al., 1991), had no effects on NGB bioelectrical properties (Fig. 5).

Inasmuch as phorbol esters have been shown to activate AC in some cells (Naghshineh, Noguchi, Huang, and Londos, 1986), we also measured the effects of



PMA (100 nM, 10 min) on the [cAMP]<sub>i</sub>. The values were  $6.1 \pm 0.8$  and  $5.9 \pm 1.0$  pmol/mg protein ( $n = 6$ ) in control conditions and after PMA, respectively. Hence, PKC activates G<sub>C1</sub><sup>a</sup> by a mechanism that does not involve changes in [cAMP]<sub>i</sub>.

#### Effects of Protein Phosphatase Inhibition

To assess the effects of PP, tissues were exposed to 8-Br-cAMP or theophylline before and after pretreatment with OA. In most experiments, tissues were exposed to OA dissolved in DMF for 30 min and then both OA and the solvent were removed before the experimental determinations (see Materials and Methods). OA (0.4 μM, 30 min, apical side) had no effects on the membrane voltages of control epithelia (see Table IV), but significantly altered R<sub>t</sub> and R<sub>a</sub>/R<sub>b</sub>; OA caused R<sub>t</sub> to increase by  $53 \pm 10 \Omega$

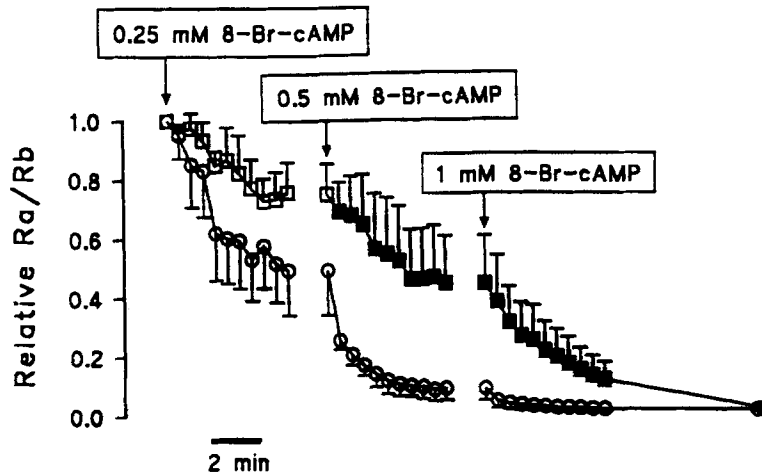


FIGURE 4. Effect of H8 pretreatment (10 μM, 60 min, apical side) on the time course of R<sub>a</sub>/R<sub>b</sub> during exposure to cumulative increases in apical solution [8-Br-cAMP]. (Circles) control. (Squares) After H8 (open squares, not significantly different from control; filled squares, significantly different from control). Data are means ± SEM ( $n =$  five paired experiments), expressed relative to R<sub>a</sub>/R<sub>b</sub> value at beginning of record. H8 slowed the responses to 8-Br-cAMP but did not affect the maximal response. See Table II.

cm<sup>2</sup> and R<sub>a</sub>/R<sub>b</sub> to fall by  $3.20 \pm 1.14$  ( $n = 17$ ). The latter results presumably denote effects of OA on the basolateral membrane (see below).

To evaluate the effects of OA alone (0.4 μM, 30 min, apical side) on the ion conductances of the apical membranes of control tissues, studies were conducted with single-ion substitutions in the apical bathing solution and K<sup>+</sup>-conductance blockers. As illustrated in Fig. 6, OA had no effects on the change in V<sub>mc</sub> upon (a) raising apical solution [K<sup>+</sup>], (b) reducing apical solution [Na<sup>+</sup>] (TMA<sup>+</sup> substitution), (c) lowering apical solution [Cl<sup>-</sup>] (cyclamate substitution), and (d) exposing tissues to K<sup>+</sup>-conductance blockers, i.e., TEA<sup>+</sup> or Ba<sup>2+</sup> (Ba<sup>2+</sup> results not shown). Note that the change in V<sub>mc</sub> upon substituting Na<sup>+</sup> with TMA<sup>+</sup> is largely due to the paracellular diffusion potential. Hence, OA has no effects on the Na<sup>+</sup>, K<sup>+</sup> or Cl<sup>-</sup> electrodiffusive permeabilities of the apical membrane. Similar results were obtained with higher [OA], i.e., 4 or 20 μM (data not shown). DMF alone had no effect on the change in

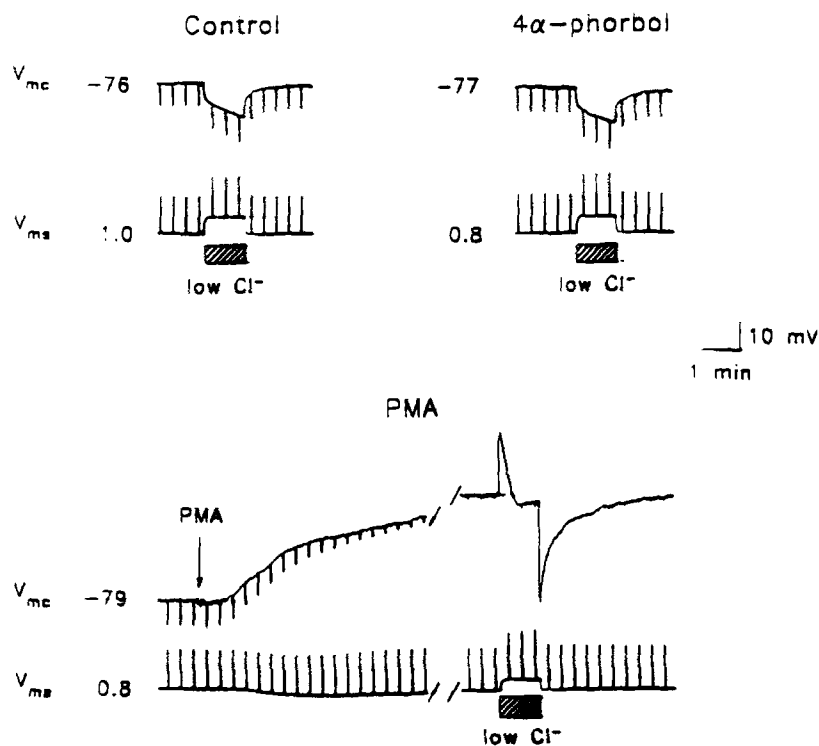


FIGURE 5. Effects of phorbol esters (100 nM, apical side) on voltages and resistances, and on changes elicited by reducing apical solution [ $Cl^-$ ]. The records are from a single tissue and are typical of results from five experiments with 4 $\alpha$ -phorbol and seven experiments with PMA. See Fig. 1 for abbreviations. (*Control*) Partial  $Cl^-$  replacement produced a slow hyperpolarization of  $V_{mc}$  consistent with the absence of a detectable  $G_{Cl}^a$ . (*4 $\alpha$ -phorbol*) Responses to reducing apical solution [ $Cl^-$ ] in the presence of 4 $\alpha$ -phorbol (phorbol ester lacking stimulatory effect on PKC) were not different from those of control. (*PMA*) PMA activated  $G_{Cl}^a$  as indicated by the consequent depolarization of  $V_{mc}$ , reduction in  $R_a/R_b$ , and characteristic responses to low  $Cl^-$ . See Table III.

$V_{mc}$  upon lowering apical solution [ $Cl^-$ ] (data not shown). To test whether OA could alter the ion conductive pathways of the apical membrane when the latter is depolarized, we carried out additional experiments with 25 mM  $K^+$  in the basolateral bathing solution. Again, OA did not alter the responses to ion substitutions in the apical bathing solution (data not shown).

TABLE III  
Effects of PMA (100 nM, Apical Side) on Voltages and Resistances

Condition	$V_{mc}$	$R_a/R_b$	$\Delta V_{mc}$
	mV		mV
Control	$-69 \pm 3$	$6.38 \pm 1.70$	—
PMA	$-48 \pm 3^*$	$0.06 \pm 0.04^*$	$18 \pm 1$

Values are means  $\pm$  SEM ( $n = 7$ ). Data are apparent steady-state values, except  $\Delta V_{mc}$ .  $\Delta V_{mc}$ , peak change in apical membrane voltage upon reduction of apical solution [ $Cl^-$ ] in the presence of PMA. See Table I for other abbreviations. \*Significantly different from paired control value.

Pretreatment with OA affected the responses to 8-Br-cAMP (1 mM, apical side) (Table IV and Fig. 7). The  $R_a/R_b$  of stimulated tissues after OA ( $0.18 \pm 0.05$ ) was significantly greater than that of stimulated tissues before OA ( $0.07 \pm 0.03$ ). Furthermore, as shown in Fig. 7, 0.4  $\mu$ M OA slowed the  $R_a/R_b$  responses to 8-Br-cAMP and hastened recovery after agonist removal. Similar results were obtained following pretreatment with higher OA concentrations (4 or 20  $\mu$ M) or in the continuous presence of 0.4  $\mu$ M OA (data not shown).

The above results are not consistent with an effect of OA on PP-catalyzed dephosphorylation of the Cl<sup>-</sup> channel responsible for  $G_{Cl}^a$ . Possible explanations include OA stimulation of PDE, as observed in rat adipocytes (Shibata, Robinson, Soderling, and Kono, 1991). As shown in Table V, OA (0.4  $\mu$ M in DMF) significantly reduced the  $[cAMP]_i$  of cells treated with 2.5  $\mu$ M forskolin. DMF alone (same concentration) did not alter  $[cAMP]_i$  or the elevation of  $[cAMP]_i$  elicited by 2.5  $\mu$ M forskolin (data not shown).

TABLE IV  
Effects of OA on Voltages and Resistances, in Absence and Presence of  
8-Br-cAMP or Theophylline

Condition	[Agonist] mM	[OA] $\mu$ M 8-Br-cAMP	$V_{mc}$ mV	$R_a/R_b$
A	0	0	$-76 \pm 2$	$6.23 \pm 1.59$
B	0	0.4	$-75 \pm 2$	$3.66 \pm 0.63^{\dagger}$
C	1	0	$-56 \pm 3^*$	$0.07 \pm 0.03^*$
D	1	0.4	$-58 \pm 3^*$	$0.18 \pm 0.05^{*\dagger}$
theophylline				
E	0	0	$-72 \pm 2$	$5.05 \pm 1.98$
F	0	0.4	$-73 \pm 3$	$2.89 \pm 0.44$
G	3	0	$-46 \pm 1^*$	$0.05 \pm 0.02^*$
H	3	0.4	$-48 \pm 4^*$	$0.04 \pm 0.03^*$

Values are means  $\pm$  SEM ( $n = 11$  for 8-Br-cAMP and 5 for theophylline). Data are apparent steady state values. See Table I for abbreviations. \*Significant effect elicited by agonist (C vs A or D vs B for 8-Br-cAMP and G vs E or H vs F for theophylline).  $^{\dagger}$ Significant effect elicited by OA (B vs. A or D vs. C for 8-Br-cAMP and F vs. E or H vs. G for theophylline).

If the effects of OA on  $G_{Cl}^a$  are mediated by stimulation of PDE, then OA should have no effects on the responses to PDE inhibitors (e.g., theophylline). As shown in Fig. 8 and summarized in Table IV, OA did not alter the responses to theophylline (3 mM, basolateral side). Hence, the effects of OA on cAMP-mediated activation of  $G_{Cl}^a$  appear to be due to stimulation of PDE. Further, OA-sensitive PP is not a prerequisite for inactivation of  $G_{Cl}^a$  following agonist removal.

#### DISCUSSION

$G_{Cl}^a$  is activated by PKA and PKC, but is insensitive to cGK

The available data indicate that PKA plays a major role in activation of  $G_{Cl}^a$  and the underlying Cl<sup>-</sup> channel. The Cl<sup>-</sup> channel can be activated in excised patches from

the apical membrane by exposure of the cytosolic membrane surface to the catalytic subunit of PKA and ATP (Copello et al., 1993). In intact tissues, channel activity and  $G_{Cl}^a$  are also stimulated by treatment with permeant cAMP analogs, forskolin or theophylline (Petersen and Reuss, 1983; Reuss and Petersen, 1985; Reuss, 1987; Copello et al., 1993; present results). Further, the present results demonstrate that

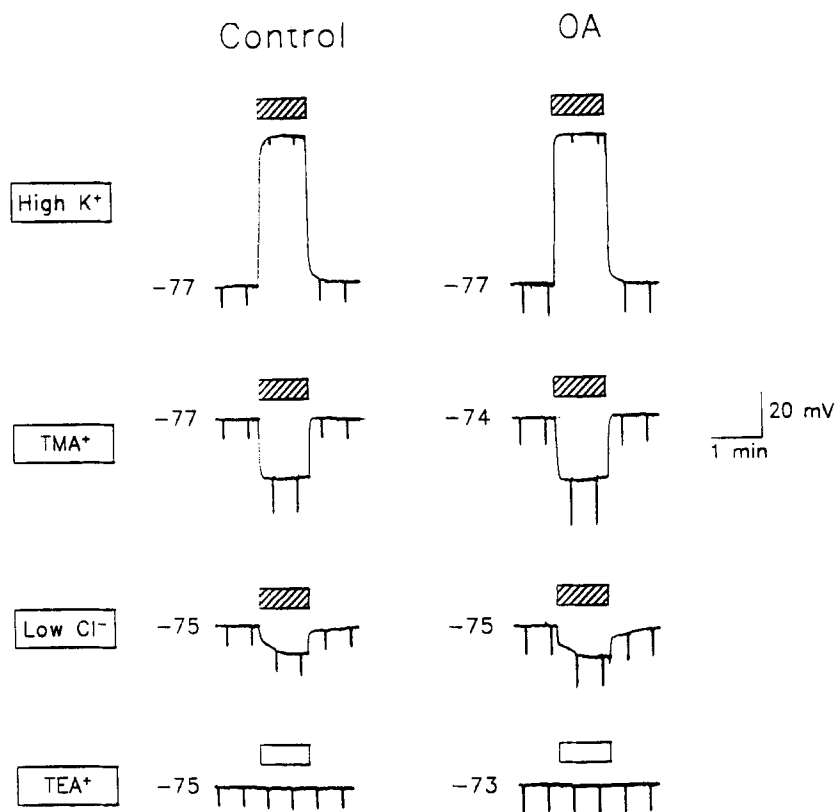


FIGURE 6. Effects of apical solution ion substitutions and  $K^+$  channel blocker on  $V_{mc}$ . Traces depict paired comparisons of responses under control conditions and after OA ( $0.4 \mu\text{M}$ , 30 min, apical side). See Fig. 1 for abbreviations. (*High  $K^+$* ) 92.5 mM, substituting for  $\text{Na}^+$ . (*TMA $^+$* ) 90 mM, substituting for  $\text{Na}^+$ . (*Low  $\text{Cl}^-$* ) 8 mM, replaced by cyclamate. (*TEA $^+$* ) 4.5 mM, substituting for  $\text{Na}^+$ . OA had no effects on the voltage changes elicited by ionic substitutions (*high  $K^+$* , *TMA $^+$* , *low  $\text{Cl}^-$* ) or the  $K^+$  channel blocker, TEA $^+$ . The larger deflections elicited by transepithelial current pulses in OA reflect the resistance changes discussed in the text, i.e.,  $R_t$  rises and  $R_a/R_b$  falls.

H8, a PKA inhibitor, slows the activation of  $G_{Cl}^a$  by 8-Br-cAMP. The H8 effect is consistent with, but does not prove, an effect of PKA on  $G_{Cl}^a$  because H8 also inhibits cGMP-dependent protein kinases (Hidaka et al., 1984).

Cyclic-GMP has been reported to modulate apical membrane  $\text{Cl}^-$  channels in epithelial cells (e.g., Lin et al., 1992). However, 8-Br-cGMP had no effects on  $G_{Cl}^a$  or

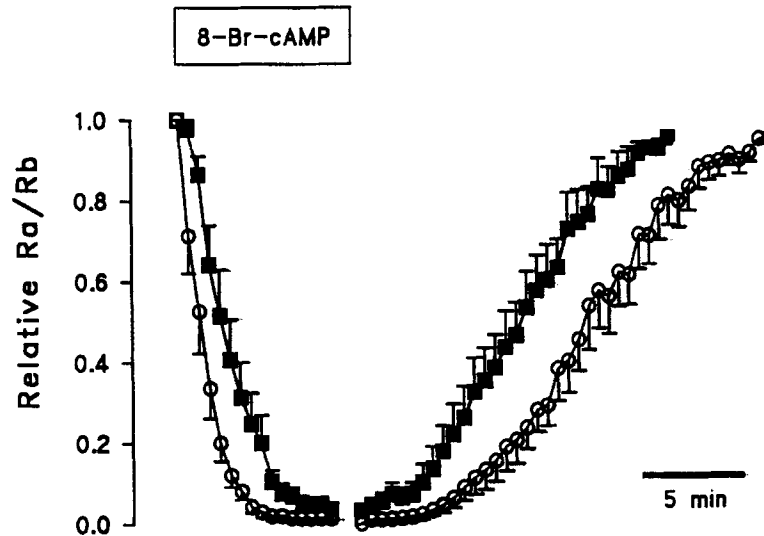


FIGURE 7. Effect of OA pretreatment (0.4  $\mu$ M, 30 min, apical side) on time course of  $R_a/R_b$  during exposure to 8-Br-cAMP (1 mM, apical side). (Circles) Control. (Squares) After OA (open squares, not significantly different from control; filled squares, significantly different from control). Data are means  $\pm$  SEM ( $n = 11$  paired experiments), expressed relative to  $R_a/R_b$  value at beginning of record. The gap in the record was 3–20 min. OA slowed  $R_a/R_b$  responses elicited by 8-Br-cAMP and hastened recovery following 8-Br-cAMP removal. See Table IV.

on the responses to forskolin. From these results, we conclude that cGMP-dependent kinases do not contribute to the regulation of  $G_{Cl}^a$ .

To investigate the possibility of activation of  $G_{Cl}^a$  by PKC, we used the phorbol esters PMA and 4 $\alpha$ -phorbol. PMA, which activates PKC (Evans et al., 1991), caused a rapid increase in  $G_{Cl}^a$  whereas the inactive analog 4 $\alpha$ -phorbol had no effects. PMA had no effects on  $[cAMP]_i$ , which rules out a cAMP-dependent mechanism of Cl<sup>-</sup> channel activation, e.g., phosphorylation of AC (Naghshineh et al., 1986). Hence, we conclude that PKC activates  $G_{Cl}^a$  via phosphorylation of the channel or a regulatory molecule, as described in HT-29cl. 19A cells (Bajnath, Van Hove, De Jonge, and Groot, 1992). PKC-induced stimulation of  $G_{Cl}^a$  does not involve an increase in

TABLE V  
Effects of OA on  $[cAMP]_i$  in the Presence of 2.5  $\mu$ M Forskolin

Time in forskolin	$[cAMP]_i$	
	Control	OA
min	<i>pmol/mg protein</i>	
0	4.7 $\pm$ 0.6	4.0 $\pm$ 0.7
2	20.8 $\pm$ 2.5	11.8 $\pm$ 2.7*
16	645 $\pm$ 84	273 $\pm$ 49*

Values are means  $\pm$  SEM ( $n = 5$ ). OA, pretreatment with 0.4  $\mu$ M okadaic acid for 30 min. \*Significantly different from paired control value. The  $[cAMP]_i$  values at 0, 2, and 16 min in each column were significantly different from each other.

$[cAMP]_i$ . Moreover, the lack of effect of ionomycin on  $G_{Cl}^a$  indicates that  $Ca^{2+}$ -independent PKC is involved.

$G_{Cl}^a$  is not activated or modulated by  $[Ca^{2+}]_i$

$Ca^{2+}$ -activated  $Cl^-$  channels play important roles in fluid secretion and cell volume regulation in epithelia (Anderson et al., 1992).  $Ca^{2+}$ -activated  $Cl^-$  channels may (e.g., Allert et al., 1992) or may not (e.g., Anderson and Welsh, 1991) possess properties similar to those of cAMP-activated  $Cl^-$  channels. In the present study, the  $Ca^{2+}$  ionophore ionomycin did not activate  $G_{Cl}^a$  although it had the expected effects on  $K^+$  conductance. We estimate the sensitivity of the  $G_{Cl}^a$  measurements as follows. The

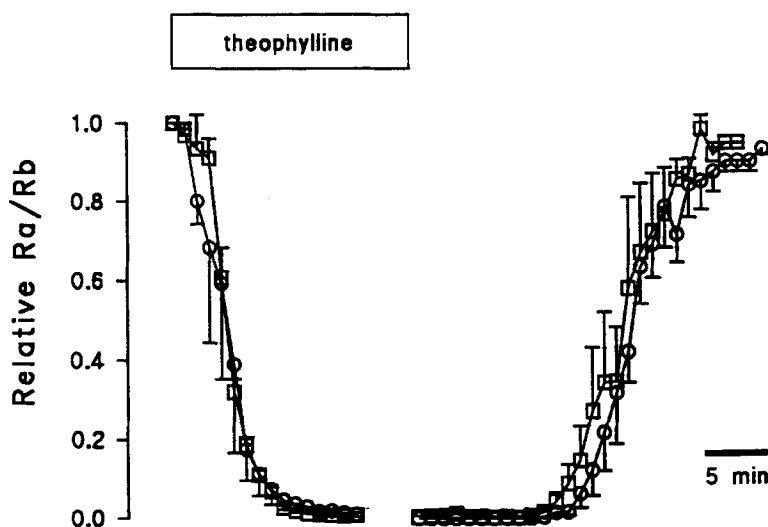


FIGURE 8. Effect of OA pretreatment ( $0.4 \mu M$ , 30 min, apical side) on time course of  $R_a/R_b$  during exposure to theophylline ( $3 mM$ , basolateral side). (Circles) Control. (Squares) After OA (not significantly different from control). Data are means  $\pm$  SEM ( $n =$  five paired experiments), expressed relative to  $R_a/R_b$  value at beginning of record. The gap in the record was 3–20 min. OA had no effects on the time course of the change of  $R_a/R_b$  after addition or removal of theophylline. See Table IV.

total apical membrane conductance ( $G^a$ ) increases  $\sim 100$ -fold during maximal cAMP stimulation (Petersen and Reuss, 1983; Copello et al. 1993; see Results). If elevating  $[Ca^{2+}]_i$  caused just 1% of the maximal cAMP effect (i.e., doubled  $G^a$ ), then  $G_{Cl}^a$  would become about 50% of  $G^a$ . Under these conditions, reducing external  $[Cl^-]$  should cause a significant apical membrane depolarization. Reducing apical solution  $[Cl^-]$  in the presence of ionomycin did not cause depolarization of  $V_{mc}$ . These results clearly indicate that  $G_{Cl}^a$  is insensitive to  $[Ca^{2+}]_i$ , in agreement with previous studies (Altenberg et al., 1992; Altenberg et al., 1993).

The present results also indicate that increasing  $[Ca^{2+}]_i$  does not affect the time course or the magnitude of the activation of  $G_{Cl}^a$  by forskolin. Previous observations

using the patch-clamp technique show that apical membrane Cl<sup>-</sup>-channel activity in excised patches is independent of the cytosolic surface [Ca<sup>2+</sup>] (Copello et al., 1993). Therefore, Ca<sup>2+</sup> is neither a direct activator nor a modulator of G<sub>Cl</sub><sup>a</sup> in NGB epithelium. Similar observations, i.e., no effect of [Ca<sup>2+</sup>]<sub>i</sub> on cAMP-activated Cl<sup>-</sup> channels, have been made by others (Anderson and Welsh, 1991; Anderson et al., 1992; Fuller and Benos, 1992; Hanrahan et al., 1993).

*OA-sensitive Protein Phosphatases are Not Involved in Inactivation of G<sub>Cl</sub><sup>a</sup>*

We envision that regulation of G<sub>Cl</sub><sup>a</sup> reflects the balance between the rate at which the Cl<sup>-</sup> conductance is activated (i.e., phosphorylation) and the rate at which it is inactivated (i.e., dephosphorylation). Phosphorylation is catalyzed by PKA, PKC, or both. Stimulation or inhibition of these protein kinases has predictable effects on G<sub>Cl</sub><sup>a</sup>. Similarly, experimental manipulation of dephosphorylation should influence G<sub>Cl</sub><sup>a</sup>. Depending on the basal rate of phosphorylation, inhibition of dephosphorylation could increase G<sub>Cl</sub><sup>a</sup>, as observed with the effects of OA on apical membrane Cl<sup>-</sup> channels of shark rectal gland epithelium (La et al., 1991). In addition, inhibition of dephosphorylation could enhance the response of G<sub>Cl</sub><sup>a</sup> to agents that promote phosphorylation, as illustrated by the effects of OA on G<sub>Cl</sub> responses of cardiomyocytes to isoproterenol or forskolin (Hwang et al., 1993). In NGB epithelium, OA did not activate G<sub>Cl</sub><sup>a</sup> by itself, nor did it enhance the G<sub>Cl</sub><sup>a</sup> response to 8-Br-cAMP. Finn et al. (1992) also found that OA had no effects on NGB G<sub>Cl</sub><sup>a</sup> by itself and did not alter the responses to theophylline. In our hands, OA reduced the G<sub>Cl</sub><sup>a</sup> responses to 8-Br-cAMP. Further, OA reduced the increase in [cAMP]<sub>i</sub> elicited by forskolin. These results are inconsistent with OA-mediated inhibition of the protein phosphatases presumably involved in inactivation of G<sub>Cl</sub><sup>a</sup> (by dephosphorylation). Rather, they suggest that OA alters the phosphorylation step, likely by stimulation of cAMP-PDE as has been demonstrated in rat adipocytes (Shibata et al., 1991). Consistent with this interpretation, the effects of OA on cAMP-mediated activation of G<sub>Cl</sub><sup>a</sup> were abolished when the latter was activated by theophylline, a PDE inhibitor.

Our studies did not include direct measurements of phosphorylation and cannot rule out the involvement of OA-sensitive PP (i.e., PP1 and PP2A) in dephosphorylation of the cAMP-activated Cl<sup>-</sup> channel or a regulatory molecule. Both a NGB Cl<sup>-</sup> channel reconstituted in lipid bilayers (Finn et al., 1992) and CFTR Cl<sup>-</sup> channels in excised patches (Berger et al., 1993) are inactivated by PP2A. The present results indicate that OA-sensitive PP do not play a major role in the inactivation of G<sub>Cl</sub><sup>a</sup> in intact NGB epithelium. Rather, they appear to modulate the activity of PDE and hence [cAMP]<sub>i</sub>, and thus influence the activation of G<sub>Cl</sub><sup>a</sup>. Hence, regulation of G<sub>Cl</sub><sup>a</sup> in intact NGB epithelium appears to be dominated by phosphorylation rather than by dephosphorylation.

*Overview of the mechanisms of control of cAMP-activated G<sub>Cl</sub><sup>a</sup> in NGB epithelium*

The data presented in this paper, in conjunction with the patch-clamp studies recently reported (Copello et al., 1993) and previous intracellular-microelectrode studies (Petersen and Reuss, 1983; Reuss and Petersen, 1985; Reuss, 1987), suggest the following general picture.

(a) Under basal conditions, the  $G_{Cl}^a$  of NGB epithelium is undetectable with intracellular-microelectrode or patch-clamp techniques.

(b)  $G_{Cl}^a$  is activated by increases in  $[cAMP]_i$  using permeant cAMP analogs, forskolin, and under some conditions theophylline. In addition,  $Cl^-$  channels in excised patches are activated by PKA plus ATP from the cytosolic surface. Finally, H8 slows the  $G_{Cl}^a$  activation elicited by 8-Br-cAMP. These results are consistent with channel activation by PKA-mediated phosphorylation.

(c)  $G_{Cl}^a$  can also be activated via PKC, by a cAMP-independent mechanism. In contrast, cGMP-dependent PK has no effects on  $G_{Cl}^a$ . These results are consistent with the pattern reported for CFTR (Berger et al., 1993).

(d) The role of protein phosphatases, i.e., of dephosphorylation, seems to be less important in controlling  $G_{Cl}^a$  than that of phosphorylation, at least with respect to okadaic acid-sensitive phosphatases.

(e) Increases in  $[G^{2+}]_i$  do not activate  $G_{Cl}^a$  or modulate the cAMP-mediated activation of  $G_{Cl}^a$ .

(f) The single-channel and whole-membrane characteristics of  $G_{Cl}^a$ , as well as its regulation, are similar to the corresponding properties of CFTR (reviewed by Anderson et al., 1992; Fuller and Benos, 1992; Hanrahan et al., 1993). The apical membrane of NGB epithelium has no detectable  $G_{Cl}^a$  in the unstimulated state and does not appear to have voltage-, or  $Ca^{2+}$  activated  $Cl^-$  conductances. Further, we have found no swelling-activated  $Cl^-$  conductances (unpublished observation). Therefore, biophysical and cell-physiologic studies of the cAMP-activated  $Cl^-$  channel might prove to be a useful model for other epithelial  $Cl^-$  channels, including CFTR.

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