J. Membrane Biol. 157, 53–61 (1997)

The Journal of
Membrane
Biology

Cross Talk of Bumetanide-sensitive and HCO_3^--dependent Transporters Activated by IBMX in Renal Epithelial A6 Cells

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Received: 9 September 1996/Revised: 6 December 1996

Abstract. We studied cAMP-dependent regulation of ion transport in aldosterone-untreated renal epithelial A6 cells by measuring short-circuit current (I_{sc}). Biphasic increases in I_{sc}, a transient phase followed by a sustained one, were elicited in response to 1 mM 3-isobutyl-1-methylxanthine (IBMX, an inhibitor of phosphodiesterase) which increased cytosolic cAMP concentration. IBMX increased the apical Cl^- conductance. The sustained phase of I_{sc} induced by IBMX was reduced by 50 μM bumetanide ($Na^+/K^+/2\ Cl^-$ cotransporter inhibitor) or 100 μM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, an inhibitor of Cl^-/HCO_3^- exchanger). Under the normal condition, the inhibitory effect of bumetanide was much larger than that of DIDS. On the other hand, under a low Cl^- condition, the effect of DIDS was more effective than that of bumetanide. Further, under a Cl^--free condition Na^+/HCO_3^- symporter contributed to the IBMX-generated I_{sc}. Taken together, our observations suggest that in A6 cells (i) IBMX stimulates Cl^- secretion associated with an increase in apical Cl^- conductances, (ii) the ionic components to generate the IBMX-induced I_{sc} are mainly maintained by bumetanide-sensitive $Na^+/K^+/2\ Cl^-$ cotransporter and DIDS-sensitive Cl^-/HCO_3^- exchanger, (iii) Cl^-/HCO_3^- exchanger coupled to Na^+/HCO_3^- symporter under a low-Cl^- condition or Na^+/HCO_3^- symporter under a Cl^--free condition contributes to the IBMX-induced I_{sc}, compensating for diminishment of the $Na^+/K^+/2Cl^-$ cotransporter-mediated Cl^- secretion, (iv) IBMX increases Cl^- and HCO_3^- conductances in the apical membrane.

Key words: Bumetanide — DIDS — Short-circuit current — cAMP — Cl^- channel

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Introduction

Differential localization of ion channels and transporters in the apical or basolateral membrane of epithelial cells is essential to vectorial ion transport such as Cl^- secretion and Na^+ absorption. For example, Na^+ absorption in distal nephron is mediated by two steps; i.e., Cl^- secretion is also by two steps; i.e., accumulation of cytosolic Cl^- by $Na^+/K^+/2Cl^-$ cotransporter or/and Cl^-/HCO_3^- exchanger in the basolateral membrane and Cl^- release through Cl^- channels in the apical membrane (Frizzell, Field & Schultz, 1979; Eaton, Marunaka & Ling, 1992). The Cl^- secretion is usually considered to be results from activation of apical Cl^- conductance (Worrell & Frizzell, 1991). In several epithelia, activation of Cl^- secretion is mediated by increased cytosolic Ca^{2+} (Van Scott & Paradiso, 1992; Dharmsathaphorn & Pandol, 1986) or cAMP (Mandel, Dharmsathaphorn & McRoberts, 1986; Johnsen & Nielsen, 1982; Greger, Schlatter & Gögelein, 1985; Greger et al., 1984).

An epithelial cell line, A6, derived from the kidney of *Xenopus laevis* is a useful model for studies of transepithelial ion transport in distal nephron (Yanase & Handler, 1986; Marunaka, Hagiwara & Tohda, 1992; De Smet & Van Driessche, 1992; Marunaka et al., 1994; Verrey, 1994; Nakahari & Marunaka, 1995; De Smet, Simaels & Van Driessche, 1995; Granitzer, Mountian & Van Driessche, 1995). The stimulatory action of antidiuretic hormone (ADH), aldosterone and cAMP on water permeability, water absorption and Na^+ transport of distal nephron epithelia is widely known (Lang, Handler & Gainer, 1986; Lang et al., 1985, 1986; Perkins & Handler 1981; Marunaka & Eaton, 1991; Verrey, 1994). In A6 cells, ADH and forskolin increase transepithelial Na^+ and Cl^- transport through cAMP-dependent signaling pathways (Chalfant, Coupaye-Gerard & Kleyman, 1993; Yanase & Handler, 1986). However, the ADH-induced

Table 1. The ionic composition of solutions used in the present study

Solution	Na	Cl	HCO_3
		(mEq/l)	
Normal	120	102.5	25
Low-Cl^-	120	7.5	25
Cl^--free	120	0	25
HCO_3^--free	120	120	0
Low-Cl^-/HCO_3^--free	120	7.5	0
Cl^-/HCO_3^--free	120	0	0
Na^+-free	0	102.5	25
Na^+/Cl^--free	0	0	25

All solutions contained (in mEq/l) 3.5 K, 1 Ca, 1 Mg, 5 glucose, 10 HEPES.
Na^+ was replaced with N-methyl-D-glucamine and choline in Na^+-free solutions.
Cl^- or HCO_3^- was replaced with gluconate in Low-Cl^-, Cl^--free, Low-Cl^-/HCO_3^--free and Cl^-/HCO_3^--free solutions.
The pH was adjusted at 7.4 with NaOH, HCl or gluconic acid before addtion of HCO_3^-.

regulation of transepithelial ion transport is not fully understood. As previously reported (Niisato & Marunaka, 1997), under a hyperosmotic condition (e.g., 270 mOsm/kg H_2O) for amphibian, the amiloride-sensitive Na^+ transport is very small and ADH cannot stimulate the amiloride-sensitive Na^+ transport. To clarify the regulation of Cl^- transport by cAMP (an intracellular second messenger of ADH), we studied the effect of cAMP on transepithelial ion transport by measuring short-circuit current (I_{sc}) under a hyperosmotic condition (270 mOsm/kg H_2O).

Materials and Methods

Chemicals

Medium NCTC-109, fetal bovine serum, penicillin, streptomycin and trypsin-EDTA were purchased from GIBCO (Grand Island, NY). 3-isobutyl-1-methylxanthine (IBMX), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid (HEPES) and nystatin were purchased from SIGMA (St Louis, MO). Bumetanide was obtained from CALBIOCHEM (San Diego, CA). All compounds were reagent grade.

Solutions

The ionic composition of solutions used in the present study is summarized in Table 1. The osmolality of solutions was approximately 270 mOsm/kg H_2O.

Cell Culture

A6 cells were purchased from American Type Culture Collection (ATCC). Briefly, A6 cells (passage 72–80) were grown on plastic flasks in NCTC-109 medium modified for amphibian cells supplemented with 10% fetal bovine serum (osmolality = 255 mOsm/kg H_2O). The flasks were kept in a humidified incubator at 26°C with 4% CO_2 in air. Cells were seeded onto polycarbonate porous membranes attached to the bottom of plastic cups (6.5 mm transwell filter; Tissue culture-treated Transwell; Costar Corporation, Cambridge, MA) at density of 1×10^5 cells/well and were cultured for 9–13 days.

Electrophysiology

Monolayers grown on polycarbonate porous membranes were rinsed with the same solution as experimental solution, and were transferred to a modified Ussing chamber (Jim's Instrument, Iowa City, IA) designed to hold the filter cup (6.5 mm transwell filter). Short-circuit currents were measured with an amplifier VCC-600 (Physiologic Instrument, San Diego, CA). A positive current represents a net flow of cation from apical to basolateral solutions or a net flow of anion from basolateral to apical solutions. Bathing solutions containing HCO_3^- were stirred with 5% CO_2/21% O_2/74% N_2. HCO_3^--free bathing solutions were stirred with 21% O_2/79% N_2. Transepithelial voltage was measured with a pair of calomel electrodes which were immersed in saturated KCl and bridged to Ussing chamber by a pair of polyethylene tubes filled with a solution of 2% agarose in 2 M KCl. To measure the transepithelial resistance, a 1.0 μA pulse (1-sec duration) was imposed across the epithelium every 10 sec. The volume of the apical and basolateral compartments was 5 ml. All experiments were performed at 22–23°C.

cAMP Assay

Before (basal) or after the cells were stimulated with 1 mM IBMX for the indicated time period, we aspirated the medium, added ice-cold 6% trichloro acetate to the cells, and kept the cells on ice for 30 min. After centrifugation at 2000 × *g* for 15 min at 4°C, the supernatants were washed 4 times with water-saturated diethyl ether. The lower aqueous extracts were lyophilized. For cAMP assay, the samples (dried extracts) were dissolved with appropriate volume of assay buffer. The concentrations of cAMP in the samples were determined with commercially available cAMP enzyme immunoassay kit (Amersham, Arlington Heights, IL).

Presentation of Data

Data are presented as the mean ± SE. The unpaired Student's *t*-test and ANOVA were used for statistical analysis as appropriate and the P value < 0.05 was considered significant.

Results

Stimulation of I_{sc} by cAMP

Biphasic increases in I_{sc} (a transient increase followed by a sustained one) were observed in response to 1 mM IBMX (an inhibitor of phosphodiesterase; applied to basolateral solution; squares in Fig. 1), which significantly increased intracellular cAMP concentrations from 232.0 ± 20.3 fmol/10^5 cells (basal) to 535.0 ± 44.7 at 3 min and

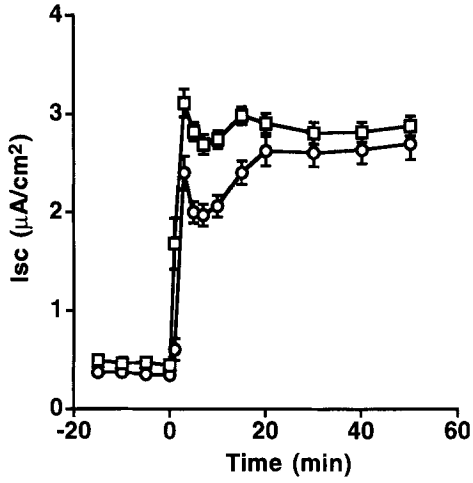

Fig. 1. Response of I_{sc} to IBMX or 8-Bromo-cAMP in Normal solution. IBMX of 1 mM (squares, $n = 8$) or 8-Bromo-cAMP of 5 mM (circles, $n = 4$) was added to the basolateral solution at time 0.

1176.7 ± 220.6 fmol/10^5 cells at 30 min after addition of IBMX ($n = 4$). I_{sc} reached a peak (transient phase) about 3 min after application of IBMX and then sustained over 50 min. The peak value of the transient increase was approximately sevenfold larger than the basal level with a decrease in resistance (Table 2). The response in I_{sc} to IBMX was mimicked by another agent which increases the intracellular concentration of cAMP; i.e., 5 mM 8-Bromo-cAMP (an analogue of cAMP) also induced biphasic increases in I_{sc} (circles in Fig. 1). IBMX increased the I_{sc} in a concentration-dependent manner (Fig. 2). These observations suggest that an increase in cytosolic cAMP concentration induces biphasic increases in I_{sc}.

Roles of Cl^- and HCO_3^- in IBMX-induced I_{sc}

To confirm roles of Cl^- and HCO_3^- in the IBMX-induced I_{sc}, we examined effects of Cl^- replacement with gluconate on the I_{sc}. In Low-Cl^- solution (7.5 mM Cl^-) with preincubation for 60 min, IBMX induced biphasic increases (triangles in Fig. 3*A*) similar to those in Normal solution (squares in Fig. 3*A*), although the sustained value of the IBMX-induced I_{sc} in Low-Cl^- solution was a little smaller than in Normal solution. On the other hand, the IBMX-induced changes in transepithelial potentials (*PD*) and resistances (R_t) were very different between those conditions (Table 3). Namely, in Normal solution R_t showed a much larger decrease in response to IBMX than in Low-Cl^- solution, while *PD* in Normal solution changed in a smaller extent in response to IBMX than in Low-Cl^- solution. Since even in Low-Cl^- solution the response of I_{sc} to IBMX was similar to Normal solution, we next completely removed Cl^- from

Table 2. Transepithelial potentials (*PD*), resistances (R_t) and short-circuit currents (I_{sc}) in standard solution

Condition		*PD*(mV)	R_t(kΩcm²)	I_{sc}(μA/cm²)
IBMX				
($n = 8$)	Base	−1.46 ± 0.17	3.45 ± 0.55	0.45 ± 0.05
	Peak	−8.02 ± 0.83	2.61 ± 0.28	3.11 ± 0.14
	Sustained	−7.23 ± 0.71	2.62 ± 0.29	2.80 ± 0.10
8-Bromo cAMP				
($n = 4$)	Base	−1.20 ± 0.08	3.15 ± 0.33	0.35 ± 0.03
	Peak	−5.90 ± 0.15	2.53 ± 0.22	2.40 ± 0.17
	Sustained	−5.62 ± 0.22	2.28 ± 0.19	2.60 ± 0.14

The data shown as peak and sustained were values at peak (about 3 min) and 30 min after addition of 1 mM IBMX or 5 mM 8-Bromo cAMP.

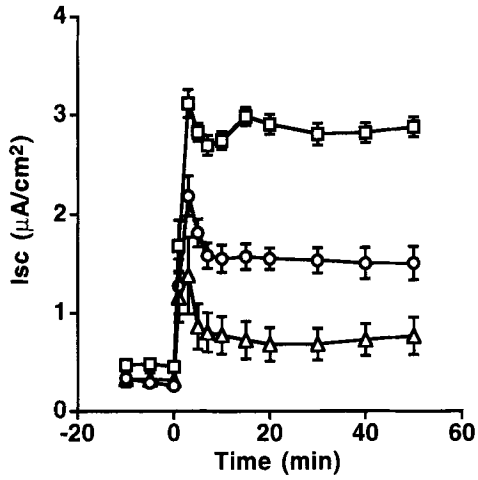

Fig. 2. Dose-dependent effects of IBMX on I_{sc}. Various concentrations of IBMX (1 mM, squares; 0.5 mM, circles; 0.1 mM, triangles; $n = 8$) were added to the basolateral solution at time 0.

bathing solution. Even in Cl^--free solution, the response of I_{sc} to IBMX (circles in Fig. 3*A*) was almost similar to that in Low-Cl^- solution (triangles in Fig. 3*A*), although some differences were observed in *PD* and R_t at the sustained phase (Table 3). We also examined the role of HCO_3^- in the IBMX-induced I_{sc}. Removal of HCO_3^- from the bathing solution (squares in Fig. 3*B*) markedly reduced both the transient and sustained phases of the I_{sc} compared with I_{sc} in Normal solution containing HCO_3^- (squares in Fig. 3*A*). Reduction of Cl^- in HCO_3^--free solution (Low Cl^-/HCO_3^--free) markedly abolished the I_{sc} at both the transient and sustained phases (triangles in Fig. 3*B*). Removal of Cl^- in HCO_3^--free solution (Cl^-/HCO_3^--free solution) completely abolished the response of I_{sc} at the sustained phase to IBMX, although a small transient increase in I_{sc} was still observed in response to IBMX (circles in Fig. 3*B*). These observations suggest that some part of the IBMX-induced I_{sc} is generated by

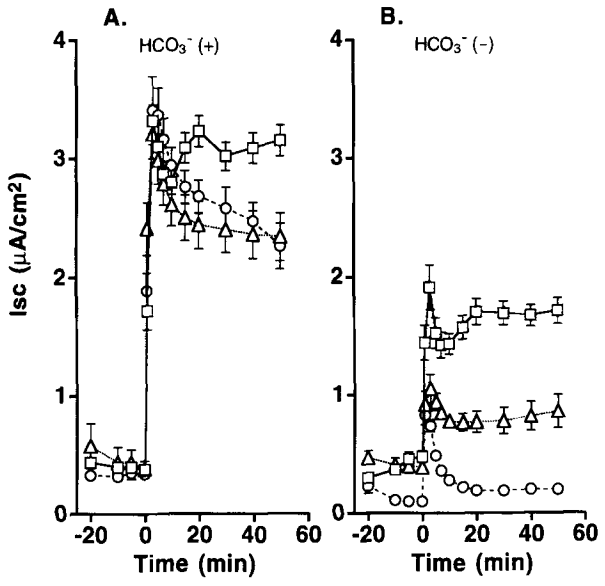

Fig. 3. Effects of Cl^- or/and HCO_3^- replacement on IBMX-induced I_{sc}. After preincubation for 60 min in each solution, 1 mM IBMX was added to the basolateral solution at time 0. (*A*) Each solution contained 25 mM HCO_3^-. Squares show Normal solution; triangles, Low-Cl^- solution; circles, Cl^--free solution. (*B*) Each solution contained 0 mM HCO_3^-. Squares show HCO_3^--free solution; triangles, Low-Cl^-/HCO_3^--free solution; circles, Cl^-/HCO_3^--free solution. n = 4–8.

Cl^-, and that removal of Cl^- is more effective in HCO_3^--free solution than HCO_3^--containing solution.

IBMX Increased Apical Membrane Conductance in Nystatin-treated Cells

To directly test effects of IBMX on the apical Cl^- conductance, we applied nystatin of 10 μM to the basolateral solution which reduced the basolateral membrane resistance by increasing monovalent ion permeability. Transepithelial resistance was stable over 30 min after the application of nystatin, while *PD* decreased during the period reaching a stable value about 30 min after application of nystatin. Addition of 1 mM IBMX to nystatin-treated cells increased the apical conductance in Normal solution (Normal Cl in Fig. 4*A*). Reduction of Cl^- from the bathing solution (Low-Cl^- solution and Cl^--free solution) markedly diminished the stimulatory effect of IBMX on the apical conductance (Low-Cl and Cl-free in Fig. 4*A*), although a small increase in conductance in response to IBMX was observed even in Low-Cl^- or Cl^--free solution. These observations suggest that most of the IBMX-induced increase in the apical conductance was due to Cl^-. Further, in HCO_3^--free solution, IBMX could increase the conductance (Normal Cl in Fig. 4*B*). Reduction of Cl^- from the bathing solution in HCO_3^--free solution (Low-Cl^-/HCO_3^--free solution and Cl^-/HCO_3^--free solution) completely abolished the IBMX-induced increase in the apical conductance (Low-Cl and Cl-free in Fig. 4*B*). Namely, the small increase in conductance induced by IBMX in Low-Cl^- or Cl^--free solution (low-Cl and Cl-free in Fig. 4*A*) was completely abolished in Low-Cl^-/HCO_3^--free or Cl^-/HCO_3^--free solution (Low-Cl and Cl-free in Fig. 4*B*), suggesting that IBMX increases HCO_3^- conductance. Taken together, these observations indicate that IBMX increases the apical Cl^- conductance and that HCO_3^- had some contribution to the IBMX-induced increase in the apical conductance.

Table 3. Transepithelial potentials (*PD*), resistances (R_t) and short-circuit currents (I_{sc}) in modified solutions

Solution	*PD*(mV)	R_t(kΩcm²)	I_{sc}(μA/cm²)
Normal			
Base	−1.46 ± 0.17	3.45 ± 0.55	0.45 ± 0.05
Peak	−8.02 ± 0.83	2.61 ± 0.28	3.11 ± 0.14
Sustained	−7.23 ± 0.71	2.62 ± 0.29	2.80 ± 0.10
Low-Cl^-			
Base	−1.77 ± 0.33	4.83 ± 0.41	0.38 ± 0.18
Peak	−14.07 ± 1.13	4.35 ± 0.38	3.21 ± 0.21
Sustained	−11.97 ± 1.49	5.00 ± 0.51	2.40 ± 0.19
Cl^--free			
Base	−1.33 ± 0.11	3.91 ± 0.23	0.34 ± 0.04
Peak	−11.04 ± 0.57	3.31 ± 0.20	3.41 ± 0.29
Sustained	−7.14 ± 1.49	2.85 ± 0.17	2.26 ± 0.19
HCO_3^--free			
Base	−0.98 ± 0.13	1.80 ± 0.26	0.48 ± 0.05
Peak	−3.22 ± 0.42	1.55 ± 0.20	1.91 ± 0.19
Sustained	−2.27 ± 0.21	1.23 ± 0.11	1.69 ± 0.10
Low-Cl^-/HCO_3^--free			
Base	−1.29 ± 0.12	3.87 ± 0.61	0.39 ± 0.06
Peak	−3.10 ± 0.45	2.94 ± 0.23	1.05 ± 0.12
Sustained	−2.50 ± 0.21	3.54 ± 0.40	0.78 ± 0.11
Cl^-/HCO_3^--free			
Base	−0.37 ± 0.11	3.35 ± 0.39	0.10 ± 0.01
Peak	−2.64 ± 0.45	3.12 ± 0.36	0.83 ± 0.08
Sustained	−0.46 ± 0.05	2.47 ± 0.43	0.20 ± 0.03

n = 4–8.

Effects of Bumetanide and DIDS on IBMX-induced I_{sc}

Our observation that a part of the IBMX-induced sustained I_{sc} was generated by Cl^- secretion (and maybe also HCO_3^- secretion) suggests that Cl^- (and maybe also HCO_3^-) is accumulated across the basolateral membrane by some mechanisms such as $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/HCO_3^- exchanger (Haas, 1994). So, using bumetanide (an inhibitor of $Na^+/K^+/2Cl^-$ cotransporter) and DIDS (an inhibitor of Cl^-/HCO_3^- exchanger), we studied roles of $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/HCO_3^- exchanger in Cl^- accumulation across the basolateral membrane. In Normal solution, two-thirds of the IBMX-stimulated I_{sc} was sensitive to bumetanide and the other

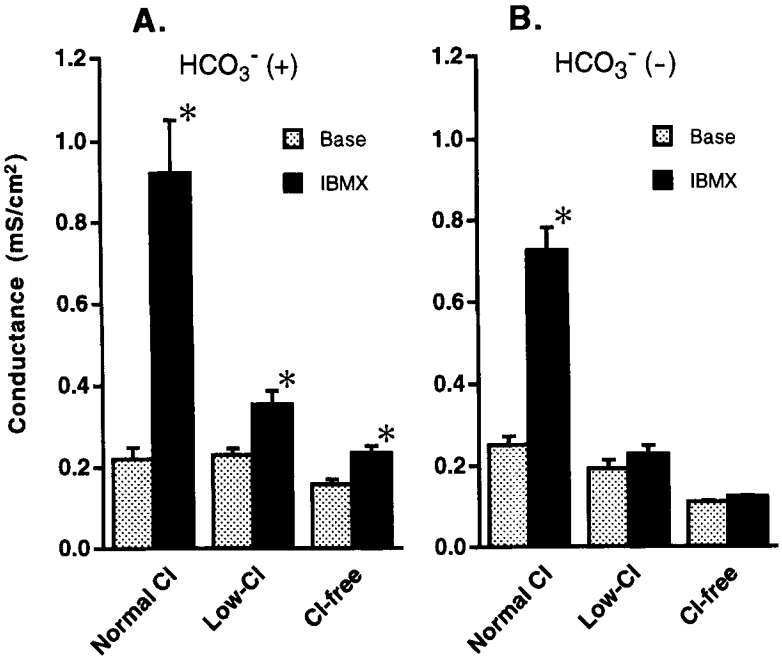

Fig. 4. Effects of Cl^- and HCO_3^- on IBMX-induced increase in apical conductances after incubation for 30 min with 10 μM nystatin in the basolateral solution. From the transepithelial resistance without (Base; IBMX (−)) and 30 min after addition of 1 mM IBMX, we calculated the basal (IBMX(−)) and IBMX-stimulated conductances. *, $P < 0.01$ compared with Base. $n = 4$. (*A*) The apical conductance with HCO_3^-. (*B*) The apical conductance without HCO_3^-.

part was mediated through DIDS-sensitive pathway (Table 4). Reduction of Cl^- (Low-Cl^- solution) diminished the bumetanide-sensitive I_{sc} but increased the DIDS-sensitive I_{sc} (Table 4). Both the bumetanide- and DIDS-sensitive components were decreased by removal of HCO_3^- from the bathing solution (HCO_3^--free solution in Table 4). On the other hand, in Low-Cl^-/HCO_3^--free solution both components were very small. These observations suggest that (i) in Normal solution, $Na^+/K^+/2Cl^-$ cotransporter mainly accumulates Cl^- and Cl^-/HCO_3^- exchanger supplementarily contributes to Cl^- accumulation, (ii) Cl^-/HCO_3^- exchanger shows a compensatory increase in its activity to accumulate Cl^- when activity of $Na^+/K^+/2Cl^-$ cotransporter is decreased by Cl^- reduction, and (iii) removal of HCO_3^- diminishes activity of both $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/HCO_3^- exchanger.

Effects of Na^+ Removal on IBMX-induced I_{sc}

Our observations suggest that Cl^-/HCO_3^- exchanger contributes to Cl^- accumulation, however we did not know how a large cytosolic electrochemical potential of HCO_3^- accumulating Cl^- was generated. One possible explanation is that HCO_3^- is accumulated by Na^+/HCO_3^- symporter. So, we tested whether removal of Na^+ from the basolateral solution abolishes the IBMX-induced I_{sc}. IBMX could only induce a small increase in I_{sc} when Na^+ was removed from the basolateral solution (the center column in Fig. 5). On the other hand, IBMX could increase I_{sc} even when Na^+ was removed from the apical solution (the right column in Fig. 5), although the increase in I_{sc} under this condition was a little smaller than that under the control condition (Normal solution shown in the left column in Fig. 5). We also tested the effect of dimethyl amiloride, a more specific blocker of Na^+/H^+ exchanger rather than Na^+ channel (Kleyman, Cragoe Jr., 1988), on the IBMX-induced I_{sc}, since vasopressin (an ADH) has been reported to activate Na^+/H^+ exchanger (Guerra et al., 1993). Dimethyl amiloride of 10 μM applied to the basolateral solution had no effect on the IBMX-induced I_{sc} under this condition, suggesting that Na^+/H^+ exchanger has no significant role in the IBMX-induced I_{sc}. Taken together, these observations suggest that the HCO_3^--dependent Cl^- accumulation across the basolateral membrane is mediated through Cl^-/HCO_3^- exchanger coupled to Na^+/HCO_3^- symporter.

Table 4. Effects of bumetanide (BMT) and DIDS on IBMX-stimulated I_{sc}

Solution	I_{sc}(μA/cm²)		
	Total	BMT-sensitive	DIDS-sensitive
Normal	2.77 ± 0.10	1.87 ± 0.13	0.88 ± 0.20
Low-Cl^-	2.40 ± 0.19	0.91 ± 0.06	1.57 ± 0.20
HCO_3^--free	1.69 ± 0.10	1.00 ± 0.03	0.35 ± 0.10
Low-Cl^-/HCO_3^--free	0.78 ± 0.11	0.17 ± 0.06	0.04 ± 0.14

Bumetanide, 50 μM; DIDS, 100 μM. Bumetanide or DIDS was added 30 min after application of 1 mM IBMX to the basolateral solution. $n = 4$–8.

Discussion

A role of cAMP in the regulation of water and solute transport has been proposed for several epithelia (Kizer

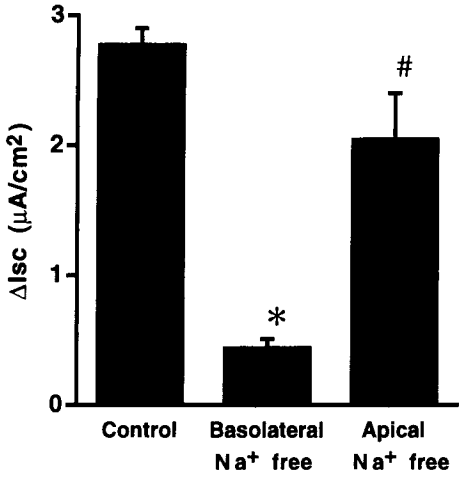

Fig. 5. The effects of Na^+ removal on the IBMX-induced I_{sc}. The removal of Na^+ from the basolateral solution (the center column; n = 8) remarkably reduced the IBMX-induced I_{sc} (*, $P < 0.001$ compared with control (n = 22)). The removal of Na^+ from the apical solution (the right column; n = 8) induced a small decrease in the IBMX-induced I_{sc} (#, $P < 0.025$ compared with control (n = 22)). HCO_3^- of 25 mM was present in the solution.

et al., 1995; Fuller, Bridges & Benos, 1994; Nagy, Naray-Fejes-Toth & Fejes-Toth, 1994). In renal epithelial A6 cells (a model of distal nephron epithelia), the ADH action is to increase Na^+ absorption. Further, cAMP, an intracellular second messenger of ADH, is known to stimulate Cl^- transport in A6 cells (Chalfant, Coupaye-Gerard & Kleyman, 1993). However, the regulatory mechanism of transepithelial ion transport by cAMP is not fully understood. In the present study, we have assessed the mechanisms of cAMP-regulated transepithelial ion transport in A6 cells. We previously reported that in A6 cells without aldosterone treatment arginine vasotocin (AVT) only induced a transient increase in I_{sc} (Doi & Marunaka, 1995*a*). In the present study, we show that even in aldosterone-untreated cells IBMX or 8-Bromo-cAMP, which is much more resistant to phosphodiesterase than dibutyryl cAMP (DB-cAMP), caused a sustained increase in I_{sc}. Our preliminary observation suggests that DB-cAMP induced only a transient increase in I_{sc} but no sustained increase. Taken together, these results suggest that the basal phosphodiesterase activity in A6 cells is very large. Further, it is suggested that a cytosolic cAMP concentration can be stably increased by IBMX or 8-Bromo-cAMP but AVT or DB-cAMP cannot stably increase or can induce only a small increase in cAMP.

We observed only a very small amount of I_{sc} generated by Na^+ absorption in the present study. The amount of the amiloride-sensitive I_{sc} in the present study was smaller than that reported in our previous study (Doi & Marunaka, 1995*a*). This difference would be due to the materials of culture; namely, we used 6.5 mm transwell filter, Tissue culture-treated Transwell (Costar Corporation), in the present study and permeable filter Falcon 3090, Cell Culture Insert, Cyclopore Membrane (Falcon) in our previous study (Doi & Marunaka, 1995*a*). Further, the small amount of amiloride-sensitive I_{sc} (Na^+ transport) is due to the osmolality of the bathing solution as shown by Wills, Millinoff and Crowe, 1991. Our previous report (Niisato & Marunaka, 1997) indicates that in a solution with 150 mOsm/kg H_2O the basal amiloride-sensitive I_{sc} was relatively large (1.67 ± 0.06 μA/cm^2, mean ± SE, n = 7), while in a solution with 270 mOsm/kg H_2O the basal amiloride-sensitive I_{sc} was very small (0.20 ± 0.02 μA/cm^2, mean ± SE, n = 17). The osmolality of the solution used in the present study was 270 mOsm/kg H_2O, indicating that the basal amiloride-sensitive I_{sc} was very small. Further, IBMX could not affect the amiloride-sensitive I_{sc} in a solution with 270 mOsm/kg H_2O (the amiloride-sensitive I_{sc} = 0.20 ± 0.02 μA/cm^2 without IBMX (mean ± SE, n = 17) and 0.22 ± 0.06 μA/cm^2 with IBMX (n = 4)). Accordingly, although there was a small amount of the amiloride-sensitive I_{sc} in the present study, the presence of the amiloride-sensitive I_{sc} could not affect the conclusion of the present study.

In general, the resistance of the basolateral membrane is much lower than that of the apical membrane. This would suggest that even if nystatin decreases the resistance of the basolateral membrane, we could not observe the effect of nystatin on the transepithelial resistance. However, it was still unclear whether under the IBMX-stimulated condition the resistance of the basolateral membrane would be much lower than that of the apical membrane. If not, nystatin applied to the basolateral membrane would have some effects on the transepithelial resistance. In the absence of IBMX, the transepithelial resistance was not significantly changed by nystatin applied to the basolateral membrane (3.45 ± 0.55 kΩ cm^2 without nystatin (mean ± SE, n = 8) and 4.99 ± 0.69 kΩ cm^2 with nystatin (n = 6)). On the other hand, in IBMX-stimulated cells, nystatin applied to the basolateral membrane significantly decreased the transepithelial resistance (2.62 ± 0.29 kΩ cm^2 without nystatin (mean ± SE, n = 8) and 1.23 ± 0.19 kΩ cm^2 with nystatin (n = 6, $P < 0.005$)). These observations suggest that under the IBMX-stimulated condition the resistance of the basolateral membrane is not much lower than that of the apical membrane, while under the basal (unstimulated) condition the resistance of the basolateral membrane is much lower than that of the apical membrane. Further, we would suggest that nystatin would really reduce the resistance of the basolateral membrane. This would also support our conclusion that IBMX increases the apical membrane conductance which depends upon Cl^- and HCO_3^-, but not upon Na^+.

Our finding that a cAMP-dependent pathway regu-

lates Cl^- secretion by activating apical Cl^- conductance is consistent with our single channel recording data that cAMP increases the open probability of 3 pS Cl^- channels and that arginine vasopressin and also cAMP increase the number of 8 pS Cl^- channels in the apical membrane (Marunaka & Eaton, 1990*a;* Marunaka & Tohda, 1993; Marunaka, 1993; Shintani & Marunaka, 1996). These observations suggest that the IBMX-induced Cl^- secretion is mediated through both types of 3 and 8 pS Cl^- channels.

We show that reduction of Cl^- concentration in 25 mM HCO_3^--containing solution had no remarkable effects on the IBMX-stimulated I_{sc}. Reduction of Cl^- concentration decreased activity of bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter but induced an increase in activity of DIDS-sensitive Cl^-/HCO_3^- exchanger, which could maintain the cytosolic Cl^- concentration. Namely, even if the cystosolic Cl^- concentration is decreased, the extent of the decrease in the cytosolic Cl^- concentration is smaller than reduction of apical Cl^- concentration. This could increase an electrochemical potential for Cl^- release across the apical membrane, resulting in maintenance of the IBMX-induced increase in I_{sc} even in Low-Cl^- solution. On the other hand, in HCO_3^--free solution reduction of Cl^- concentration remarkably decreased the IBMX-induced I_{sc}. This would be caused by diminution of Cl^- accumulation due to abolishment of activity of both $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/HCO_3^- exchanger. Taken together, even though reduction of bathing Cl^- concentration decreases cytosolic Cl^- concentration, upregulated Cl^-/HCO_3^- exchanger maintains cytosolic Cl^- concentration, resulting in an increase in the electrochemical potential for Cl^- release across the apical membrane, which can keep Cl^- secretion at a level similar to that in Normal solution. Further, under Cl^--free condition the IBMX-induced I_{sc} is generated by HCO_3^- secretion via accumulation by Na^+/HCO_3^- symporter across the basolateral membrane and HCO_3^- release through HCO_3^--permeable anion channel at the apical membrane.

We should argue the reason why removal of HCO_3^- decreases the bumetanide-sensitive component of the IBMX-induced I_{sc}. Our previous report (Doi & Marunaka, 1995*b*) indicates that in the absence of HCO_3^-, cAMP decreases cytosolic pH by inhibiting Na^+/H^+ exchanger. On the other hand, a decrease in cytosolic pH inhibits $Na^+/K^+/2Cl^-$ cotransporter (Raat et al., 1994). These observations indicate that activity of cAMP-activated bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter is reduced by removal of HCO_3^- through cAMP-induced cytosolic acidification. So, removal of HCO_3^- diminished the stimulatory action of cAMP not only on Cl^-/HCO_3^- exchanger but also on $Na^+/K^+/2Cl^-$ cotransporter through cytosolic acidification.

We conclude that accumulation of cytosolic Cl^- across the basolateral membrane is mediated by $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/HCO_3^- exchanger. However, it is still unclear whether those ion transporter systems are activated by cAMP. Recently, phosphorylation of $Na^+/K^+/2Cl^-$ cotransporter has been shown to increase its activity (Haas, 1994). Further, Cl^-/HCO_3^- exchanger has also been reported to be activated by protein kinase A (PKA)-dependent phosphorylation (Clari, Bordin & Moret, 1992; Baggio et al., 1993; Yannoukakos et al., 1991). So, in A6 cells these ion transporters would be activated by PKA-dependent phosphorylation. The apical membrane of A6 cells has a Ca^{2+}-activated 3 pS Cl^- channel (Marunaka & Eaton, 1990*a;* Marunaka & Eaton, 1990*b*). If the IBMX-induced sustained phase of I_{sc} does not require stimulation of cytosolic Cl^- accumulation, only an increase in the apical Cl^- conductance can cause a sustained increase in I_{sc}. Our observation (Niisato & Marunaka, 1996) that ionomycin, which can activate Ca^{2+}-activated 3 pS Cl^- channel (Marunaka & Eaton, 1990*a,b*) by increasing the cytosolic Ca^{2+} concentration, induces only a transient increase in I_{sc} without any sustained increase. Therefore, in A6 cells the IBMX-induced sustained phase would require activation of $Na^+/K^+/2Cl^-$ cotransporter or/and Cl^-/HCO_3^- exchanger, suggesting that IBMX activates $Na^+/K^+/2Cl^-$ cotransporter or/and Cl^-/HCO_3^- exchanger.

Kleyman and his colleagues (Chalfant, Coupaye-Gerard & Kleyman, 1993) have reported that reduction of Cl^- concentration almost completely blocks a stimulatory action of arginine vasopressin on Cl^- secretion. They completely removed Cl^- from bathing solutions and low concentration of HCO_3^- (2.5 mM). This is consistent with our observation shown in the present study. Further, we could not observe a stimulatory effect of IBMX on amiloride-sensitive I_{sc}, but Kleyman and his colleagues (Chalfant, Coupaye-Gerard & Kleyman, 1993) have shown that arginine vasopressin increases an amiloride-sensitive I_{sc}. These different observations would be due to the different conditions; we used A6 cells without aldosterone treatment, while Kleyman and his colleagues (Chalfant, Coupaye-Gerard & Kleyman, 1993) have used aldosterone-treated A6 cells.

In epithelial tissues, some studies indicate that cell volume is decreased by cAMP (He et al., 1989; MacLeod, Lembessis & Hamilton, 1994; Chen et al., 1994; Nakahari & Marunaka, 1996), whose concentration is elevated by IBMX. If cell volume decreases in epithelial monolayer, the intercellular space would increase resulting in a decrease in the shunt (paracellular) resistance. This means that cAMP would increase paracellular ionic conductances. On the other hand, to study the role of Na^+ in the IBMX action, we removed Na^+ in the basolateral or apical solution. The removal of basolateral Na^+ generates the Na^+ gradient inducing cation movements from the apical to the basolateral side even

under the short circuit condition. As mentioned above, IBMX would induce an increase in the paracellular ionic conductance decreasing cell volume. As shown in Fig. 5, in a basolateral Na^+-free solution IBMX induced a small increase in the I_{sc}. Accordingly, this IBMX-induced small increase in I_{sc} would be due to an increase in cation movements from the apical to the basolateral side caused by an increase in the paracellular ionic conductance, but not due to an increase in the transcellular Cl^- release. On the other hand, the removal of apical Na^+ generates the Na^+ gradient inducing cation movements from the basolateral to the apical side. If IBMX increases the paracellular ionic conductance by reducing cell volume, the cation movements from the basolateral to the apical side through the paracellular ionic conductive pathway could increase. This increase in cation movement through the paracellular ionic conductive pathway would diminish the IBMX-induced increase in I_{sc}. The IBMX-induced increase in I_{sc} in the apical Na^+-free solution was smaller than that in control (Fig. 5). Accordingly, the smaller increase in the IBMX-induced I_{sc} in an apical Na^+-free solution compared with in control would be due to an increase in cation movement through the paracellular ionic conductive pathway, but not due to a decrease in the transcellular Cl^- release.

The removal of Na^+ from the basolateral solution may have some effects in addition to blocking effects on $Na^+/K^+/2Cl^-$ cotransporter and Na^+/HCO_3^- symporter; for instance, the Na^+ removal would abolish the activity of Na^+/H^+ and Na^+/Ca^+ exchanger. Our observation that dimethylamiloride (10 μM), a specific inhibitor of Na^+/H^+ exchanger, had no significant effects on the I_{sc}, suggesting that the Na^+/H^+ exchanger would have no significant function at least in the presence of HCO_3^-. However, it is still unclear whether the removal of Na^+ affects the cytosolic Ca^{2+} concentration. In general, the basolateral membrane has Na^+/Ca^+ exchanger. The removal of Na^+ may increase the cytosolic Ca^{2+} concentration by abolishing the activity of Na^+/Ca^+ exchanger. Our observation (Niisato & Marunaka, 1996) suggests that IBMX would increase I_{sc} by elevating the cytosolic Ca^{2+} concentration through an increase in Ca^+ influx. Accordingly, the removal of Na^+ could affect the basal and IBMX-induced I_{sc} by elevating the cytosolic Ca^{2+} concentration. More experiments related to the action of cytosolic Ca^{2+} on I_{sc} may be required for complete study on the effects of Na^{2+} removal from the basolateral solution.

In summary, we conclude that (i) A6 cells have high activity of phosphodiesterase; (ii) IBMX increases apical Cl^- conductances; (iii) IBMX activates $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/HCO_3^- exchanger; (iv) reduction of $Na^+/K^+/2Cl^-$ cotransporter activity by low Cl^- induces an increase in Cl^-/HCO_3^- exchanger activity as compensation; (v) Na^+/HCO_3^- symporter contributes to Cl^- accumulation with Cl^-/HCO_3^- exchanger. We propose a model of Cl^- secretion in A6 cells stimulated by cAMP (Fig. 6).

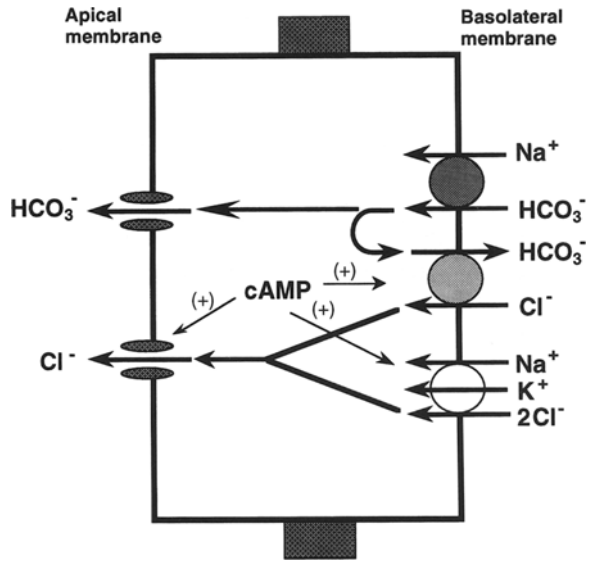

Fig. 6. A model of Cl^- secretion in A6 cells stimulated by cAMP.

This work was supported by a Grant-in-Aid from the Kidney Foundation of Canada to Y. Marunaka and partly supported by Grants-in-Aid from the Medical Research Council of Canada (Group Grant, Project 9 and MA13494), the Ontario Thoracic Society (Block Term Grant), MPG Research Fund to Y. Marunaka, and the Ministry of Education, Science and Culture of Japan (#07044298). The authors thank Prof. W. Van Driessche for critically reading the manuscript and useful suggestions. Y. Marunaka is a Scholar of the Medical Research Council of Canada.

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