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pH modulates cAMP-induced increase in Na^+ transport across frog skin epithelium

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Abstract

Apical membrane potential (V_a), fractional apical membrane resistance (FR_a), and/or intracellular pH (pH_i) were measured in principal cells of isolated frog (*Rana pipiens*) skin with microelectrodes under short-circuit conditions. Apical exposure to 0.33 mM 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (cAMP) depolarized V_a, decreased FR_a and increased short-circuit current (I_{sc}). cAMP-induced 50% larger effects on V_a and I_{sc} at external pH (pH_o) of 8.0 than at pH_o 6.4. Increasing pH_o from 6.4 to 8.0 in presence of cAMP further depolarized V_a and increased I_{sc}. cAMP-induced effects on V_a and I_{sc} were observed in the absence of Cl^- and HCO_3^- and in the presence of 1 mM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) or 10 μM 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) or 1 μM 5-(*N*-methyl-*N*-isobutyl)amiloride (MIA). These data indicate that Na^+-H^+ exchange, Cl^--HCO_3^- exchange, and electrogenic Na^+-$(HCO_3^-)_n$ cotransport are not involved in cAMP-induced increase in I_{sc}. Apical exposure to 2 mM Cd^{2+} or Zn^{2+} depolarized V_a, decreased FR_a, increased I_{sc} and increased pH_i. In HCO_3^--free solutions containing DIDS, unilateral replacement of apical Cl^- by NO_3^- induced a fast transient depolarization of V_a and an increase in I_{sc}. These data suggest that potential-dependent changes in pH_i are involved in increases in I_{sc}. However, when changes in V_a were minimized by pretreating the basolateral membrane with 25 or 75 mM K^+, the cAMP-induced increase in I_{sc} was not blocked. These data indicate that changes in pH_i do not play a strict regulatory role but are only permissive in cAMP-induced effects on I_{sc}.

Keywords: Adenosine 3',5'-cyclic monophosphate; pH, intracellular; pH-sensitive microelectrode; Short-circuit current; Potential-induced change in pH_i; (Frog skin epithelium)

1. Introduction

Changes in both extracellular pH (pH_o) and intracellular pH (pH_i) modulate the action of a variety of hormones [13,32,33]. Application of arginine vasotocin (AVT)- or cyclic 3',5'-adenosine monophosphate (cAMP) to frog skin epithelium increases overall transepithelial Na^+ transport, measured as short-circuit current (I_{sc}) [19]. In our previous studies with pH-sensitive double-barrelled microelectrodes [30] we observed that this increase in I_{sc} was accompanied by depolarization of apical cell membrane potential (V_a), a decrease in fractional resistance of the apical cell membrane (FR_a) and an increase in pH_i in principal cells. There was a strong temporal relationship between AVT- or cAMP-induced initial increase in pH_i and I_{sc}. These data indicate that changes in pH_i are involved in the action of antidiuretic hormone (ADH [1]). Changes in pH_o and/or pH_i can modulate ADH action by altering its binding to the receptor [23,24], modulation of ADH-induced increase in intracellular cAMP [23,24], and mobilization of intracellular Ca^{2+}, $[Ca^{2+}]_i$ [23–25]. ADH-induced changes in pH_i can affect transepithelial Na^+ transport by direct effects on amiloride-sensitive apical Na^+ channels [4,20,29,38], basolateral Na^+-K^+-ATPase [11], and incorporation of channels from intracellular stores into the apical cell membrane [1,32,49].

However, we have not investigated the mechanisms by which ADH and/or cAMP induce an increase in pH_i. In this paper we have evaluated the relative role of Na^+-H^+ exchange, Cl^-–HCO_3^- exchange, electrogenic Na^+-

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[1] Antidiuretic hormone (ADH) is synonymous for arginine vasopressin (AVP), vasopressin (VP), arginine vasotocin (AVT), and oxytocin.

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$(HCO_3^-)_n$, and changes in membrane potential upon pH_i changes in principal cells which do not accomplish these changes in pH_i through the action of ion pumps [8,9,12,21,28,29,31,32]. Secondly, we examined whether changes in pH_i play a permissive or a strict obligatory role in cAMP action. To achieve this objective, we studied the effect of cAMP on I_{sc}, V_a and/or pH_i in principal cells of frog skin under various experimental conditions in which one or more pH_i regulatory mechanisms are either activated or blocked.

The frog skin has long served as an important model of mammalian epithelial cell function and hormone regulation. The essential findings of this study are that changes in pH_o and/or pH_i modulate the cAMP effects on overall Na^+ transport (measured as I_{sc}). A decrease in pH_o and/or pH_i attenuates while an increase in pH_o and/or pH_i augments cAMP-induced increase in I_{sc}. The cAMP-induced increase in I_{sc} does not involve the participation of Na^+-H^+ exchanger, Cl^--HCO_3^- exchanger or the electrogenic Na^+-$(HCO_3^-)_n$ cotransporter, but is temporally related to the potential-induced increase in pH_i. These data will help to focus future studies on the precise mechanism whereby changes in membrane potential affect pH_i, in particular leak pathways involving Na^+ channels [31]. Since changes in pH_o and/or pH_i modulate both basal as well as cAMP-induced increase in I_{sc}, in a number of pathological conditions changes in pH may have significant effects in health and disease. A decrease in luminal pH in the kidney distal tubule may attenuate both basal as well as hormone-induced Na^+ transport. An increase in pH may result in a greater basal Na^+ conductive transport and a greater sensitivity to hormonal stimulation. Therefore, under chronic acidosis or alkalosis the hormonal regulation of Na^+ conductive transport may be seriously compromised [32].

Parts of these data have been reported in abstract form (Lyall, V. and Biber, T.U.L. (1994) FASEB J. 8, A348 (Abstr. 2016).

2. Materials and methods

The methods for mounting the tissues and for voltage clamping have been described [8,9,28–30]. In brief, *Rana pipiens* of the Southern variety (Rana Laboratories, Brownsville, TX) were sacrificed by double pithing and circular pieces of skin were mounted with the apical surface facing upwards in a chamber designed to avoid edge damage. The apical and basolateral surfaces of the isolated epithelium were perfused separately. The perfusion chamber was connected to an automatic voltage clamping device (Physiologic Instruments, San Diego, CA) that permitted correction for fluid resistance and determination of slope resistance (R_t) and of fractional resistance of the apical cell membrane (FR_a) by imposition of square wave transepithelial (V_t) pulse. All measurements were made with respect to the apical bathing solution.

Table 1
Composition of Ringer's solutions (in mM)

	A	B	C	D	E	F
NaCl	110.0	110.0	110.0		87.5	37.5
KCl		2.5			22.5	72.5
$CaCl_2$	1.0	1.0	1.0		1.0	1.0
KOH			2.5	2.5		
NaOH		2.5				
Hepes		3.3	3.3	3.3		
$KHCO_3$	2.5				2.5	2.5
$NaNO_3$				110.0		
$Ca(NO_3)_2$				1.0		
CO_2 (%) [a]	0.2				0.2	0.2
O_2 (%)		100	100	100		

[a] Balance air. Unless otherwise stated the pH of Ringer's solutions was maintained at 8.0. In some experiments the pH of solution B was adjusted to 6.4. In experiments with divalent metal ions the pH of solution C was adjusted to 7.4.

The composition of the six types of frog Ringer's solutions (A to F) used in this study is given in Table 1. In some Ringer's solutions Cl^- and HCO_3^- were replaced by NO_3^- and Hepes, respectively. Unless otherwise mentioned the pH of Ringer's solutions were maintained at 8.0. Ringer's solutions containing 2.5 mM HCO_3^- were continuously bubbled with 0.2% CO_2/air mixture. Solutions without HCO_3^- were bubbled with 100% O_2. Liquid junction potentials between Ringer's solution C and Ringer's solution D (Table 1) were measured as described by Garcia-Diaz et al. [15] and were found to be less than 1.5 mV. After mounting, the tissues were allowed to equilibrate for at least 30 min before any steady-state electrical measurements were recorded.

In our initial studies we used single-barreled conventional microelectrodes to monitor changes in V_a. The microelectrodes were pulled from Kwik-fil glass capillaries (1B120F; World Precision Instruments, Sarasota, FL) and were backfilled with 150 mM KCl; their resistances were about 150 MΩ. In additional studies we monitored pH_i with double-barreled pH-sensitive microelectrodes. The technique for manufacturing and using such microelectrodes have been described [8,28–30]. In this study, the pH-sensitive microelectrodes had slopes of -54 ± 1 mV (means $\pm$ S.E., $n = 23$) and the resistance of the reference barrel was typically around 120 MΩ.

The electrical potential difference across the apical cell membrane (V_a) determined by the reference barrel and the potential determined by the ion-selective barrel were measured with reference to the apical bathing solution via two high-input resistance (10^{15} Ω) electrometers (FD223, World Precision Instruments, Sarasota, FL). All signals, including I_{sc} and V_t were digitally recorded and simultaneously fed to a strip-chart recorder (2400S, Gould, Cleveland, OH) as well as to digital meters for continuously

monitoring their values. Details of the criteria used to determine the acceptability of impalements have been described previously [7]. All measurements were made in short-circuited skin preparations.

Amiloride (Merck Sharp and Dohme, West Point, PA) was used at 0.1 mM. 5-(*N*-methyl-*N*-isobutyl)amiloride (MIA) was obtained from Research Biochemicals, Natic, MA, and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) was purchased from Molecular Probes, Eugene, OR. MIA was used at 1 μM and EIPA was used at 10 μM. Stock solutions of MIA and EIPA were prepared in 0.1 N HCl and ethanol, respectively. In experiments with EIPA an equivalent amount of ethanol was added to control solutions. DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; Sigma, St. Louis, MO) was used at 1 mM. HCO_3^--free Ringer's solutions were buffered with Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Sigma). 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate (8-CPT-cAMP; Sigma) was used at 0.33 mM. In our previous studies, application of apical 8-CPT-cAMP produced rapid and completely reversible effects on V_a, I_{sc}, pH_i and FR_a in skin preparations [30]. The same experimental protocol was used in this study. In some experiments the apical surface of frog skin was exposed to 2 mM $ZnCl_2$ or $CdCl_2$ (Sigma).

Numerical values are presented as mean values ± S.E. These are followed by the number of tissues involved. Student's *t*-test was employed to analyze the significance of observed differences between sets of data. All comparisons were made between paired measurements. Linear regression coefficient is denoted as '*r*'.

3. Results

3.1. Studies with single-barreled conventional microelectrodes

3.1.1. Effects of cAMP in absence of Cl^- and HCO_3^-

Skin preparations were perfused on both sides with Cl^--HCO_3^--free Ringer's solution (Ringer's solution D; pH 8.0; Table 1) for 60 min. After a stable impalement apical solution was switched to a solution containing 0.33 mM 8-CPT-cAMP. In three other experiments tissues were perfused on both sides with Ringer's solution D (Table 1) containing, in addition, 1 mM DIDS, an inhibitor of the Cl^-–HCO_3^- and Cl^-–OH^- exchangers. Since no qualitative differences were observed in cAMP effects in Cl^--HCO_3^--free solutions with and without DIDS, the pooled data from these experiments is summarized in Table 2. In 10 tissues cAMP depolarized V_a by 29 mV and increased I_{sc} by 49 μA. Although these changes were accompanied by a small decrease in FR_a, the overall decrease in FR_a was not statistically significant. As reported earlier [30] the cAMP induced effects on V_a, FR_a and I_{sc} were completely blocked by 0.1 mM apical amiloride. These data suggest that Cl^-–HCO_3^- exchanger, Cl^-–OH^- exchanger and electrogenic Na^+-$(HCO_3^-)_n$ cotransport are not involved in cAMP effects on V_a and I_{sc}.

Table 2
Effect of cAMP in the complete absence of Cl^- and HCO_3^-

Apical cAMP	V_a (mV)	I_{sc} (μA)	FR_a
−	−56.2±4.6	29.4±4.4	0.51±0.08
+	−27.2±2.9	78.6±2.8	0.40±0.04
Δ	29.0±5.3	49.2±4.6	−0.11±0.08
P (paired)	< 0.001	< 0.001	NS

Values are means ± S.E. of $n = 10$. Skin preparations were perfused with Ringer's solution D (Table 1; pH 8.0) with ($n = 3$) or without ($n = 7$) 1 mM DIDS. During a stable impalement the apical solution was changed to Ringer's solution D + 0.33 mM 8-CPT-cAMP.

3.1.2. Effects of cAMP in presence of EIPA or MIA

The Na^+–H^+ exchanger was inhibited by exposing the tissue for 30 min on both sides to either 10 μM EIPA ($n = 12$) or 1 μM MIA ($n = 6$). Since the effects of cAMP in the presence of both EIPA and MIA were found to be qualitatively similar, the data from the two treatments was pooled. As shown in Table 3, in 18 tissues bathed on both sides in HCO_3^--free solution (Ringer's solution C; pH 8.0; Table 1) containing either EIPA or MIA, cAMP increased I_{sc} by 31 μA. In 12 tissues impaled with single-barreled microelectrodes cAMP depolarized V_a by 33.9 mV and decreased FR_a by 0.26. These data suggest that Na^+-H^+ exchange is not involved in cAMP-induced effects on I_{sc} and V_a.

3.1.3. Effects of cAMP in presence of basolateral high K^+

The potential-induced changes in pH_i can be blocked by preventing changes in V_a [28,29,31]. In the next series of experiments cAMP-induced changes in V_a were minimized by clamping V_a to different values by pretreating the basolateral surface of frog skin with high concentrations of K^+. Such an experiment is shown in Fig. 1. In agreement with our previous studies [28], in a tissue bathed on both sides with Ringer's solutions A (Table 1; pH 8.0), increasing basolateral K^+ concentration from 2.5 to 25 mM (Ringer's solution E; Table 1) depolarized V_a from

Table 3
Effect of cAMP in the presence of MIA or EIPA

Apical cAMP	V_a (mV)	I_{sc} (μA)	FR_a
−	−80.2±4.0	21.7±2.5	0.6±0.02
+	−46.3±3.4	52.3±4.1	0.39±0.04
Δ	33.9±2.2	30.6±4.0	−0.26±0.03
P (paired)	< 0.001	< 0.001	< 0.001
n	12	18	12

Values are means ± S.E. of *n*. Tissues were perfused with Ringer's solution C (Table 1; pH 8.0) containing, in addition, either 10 μM EIPA ($n = 12$) or 1 μM of MIA ($n = 6$). During a stable impalement apical solution was switched to Ringer's solution C + EIPA or MIA + 0.33 mM 8-CPT-cAMP.

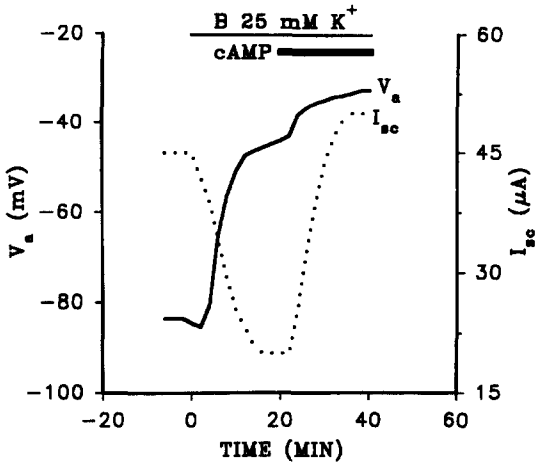

Fig. 1. Effect of 0.33 mM apical cAMP in the continuous presence of 25 mM basolateral K^+. Solid thick line represents V_a and the dotted line represents I_{sc}. Thin horizontal bar on top of the figure (labelled as B 25 mM K^+) represents the time period during which the basolateral membrane (B) was exposed to 25 mM K^+. Thick horizontal bar (labelled as cAMP) represents the time period during which the apical cell membrane was exposed to cAMP.

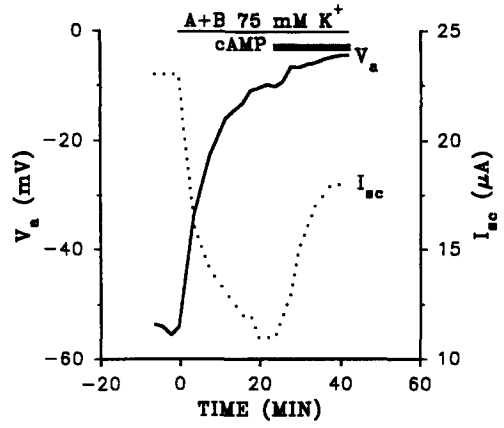

Fig. 2. Effect of 0.33 mM apical cAMP in the continuous presence of bilateral 75 mM K^+. Solid thick line represents V_a and the dotted line represents I_{sc}. Thin horizontal bar on top of the figure (labelled as A + B 75 mM K^+) represents the time period during which the skin was bilaterally (A + B) exposed to 75 mM K^+. Thick horizontal bar (labelled as cAMP) represents the time period during which the apical cell membrane was exposed to cAMP.

−83.5 to −45.4 mV ($\Delta V_a = 38.1$ mV) and decreased I_{sc} from 45 to 20 μA ($\Delta I_{sc} = -25$ μA). In continuous presence of 25 mM basolateral K^+ (Ringer's solution E), 0.33 mM cAMP induced a depolarization of V_a from −45.4 to −32.6 mV ($\Delta V_a = 12.8$ mV) and increased I_{sc} from 20 to 50 μA ($\Delta I_{sc} = 30$ μA). The initial changes in V_a (ΔV_a) and I_{sc} (ΔI_{sc}) proceeded concurrently. The most rapid changes in V_a and I_{sc} occurred in the first 2.5 min after cAMP application and within that time ΔI_{sc} was strictly proportional to ΔV_a ($r = 0.98$; $n = 12$). This time-course of cAMP-induced changes in V_a and I_{sc} is similar to that observed earlier by us [30]. As summarized in Table 4, in 5 tissues treated with 25 mM basolateral K^+, cAMP depolarized V_a by 10.4 ± 1.6 mV and increased I_{sc} by 26.0 ± 2.2 μA.

In Fig. 2, the tissue was pretreated with 75 mM K^+ (Ringer's solution F; Table 1) on both apical and basolateral surfaces. Under these conditions there are no ion gradients across the tissue. Bilateral 75 mM K^+ depolarized V_a from −53.6 to −9.2 mV ($\Delta V_a = 44.4$ mV) and decreased I_{sc} from 23 to 11 μA ($\Delta I_{sc} = -12$ μA). Under these conditions, apical cAMP depolarized V_a from −9.2 to −4.5 mV ($\Delta V_a = 4.7$ mV) and increased I_{sc} from 11 to 18 μA ($\Delta I_{sc} = 7$ μA). Once again cAMP-induced initial changes in V_a and I_{sc} occurred concurrently. Data summa-

Table 4
Effect of cAMP in the presence of high K^+ concentrations

Apical cAMP	K^+ (mM) apical/basolateral	V_a (mV)	I_{sc} (μA)	FR_a
−	2.5/2.5	−88.7 ± 4.1	28.8 ± 6.4	0.70 ± 0.06
−	2.5/25.0	−39.2 ± 2.1	13.2 ± 2.9	0.86 ± 0.03
	Δ_1	49.5 ± 4.4 ***	−15.6 ± 3.8 *	0.16 ± 0.04 **
+	2.5/25.0	28.9 ± 3.1	39.2 ± 4.4	0.66 ± 0.03
	Δ_2	10.4 ± 1.6 ***	26.0 ± 2.2 ***	−0.21 ± 0.04 **
−	2.5/ 2.5	−71.9 ± 10.1	25.5 ± 1.4	0.75 ± 0.07
−	75.0/75.0	−17.2 ± 3.5	11.5 ± 0.3	0.99 ± 0.02
	Δ_3	54.7 ± 9.0 *	−14.0 ± 1.7 **	0.24 ± 0.04 *
+	75.0/75.0	−13.2 ± 3.9	17.0 ± 0.6	0.75 ± 0.05
	Δ_4	4.0 ± 0.4 ***	5.5 ± 0.3 ***	−0.16 ± 0.03 *

Values are means ± S.E. of n. * $P < 0.05$; ** $P < 0.025$; *** $P < 0.01$. In the first set of experiments Δ_1 and Δ_2 represent paired differences from $n = 5$. In the basolateral compartment Ringer's solution A was unilaterally switched to basolateral Ringer's solution E (Table 1; pH 8.0). After reaching a new steady-state in 25 mM basolateral K^+, the apical solution was changed to Ringer's solution A + 0.33 mM 8-CPT-cAMP.In the second set of experiments Δ_3 and Δ_4 represent paired differences from $n = 4$. Ringer's solution A was bilaterally switched to Ringer's solution F (Table 1; pH 8.0). After reaching a new steady-state in the presence of 75 mM K^+, the apical solution was changed to Ringer's solution F + 0.33 mM 8-CPT-cAMP.

Table 5
Effect of pH_o on cAMP action

Apical cAMP	V_a (mV)	I_{sc} (μA)
pH_o 6.4		
−	−72.9±9.3	12.7±3.1
+	−55.7±9.1	15.5±3.0
Δ	17.2±3.9	20.0±7.8%
P (paired)	< 0.05	NS
n	4	4
pH_o 8.0		
−	−47.4±1.5	15.4±2.4
+	−17.2±6.7	22.6±2.9
Δ	30.2±8.0	46.8±7.2%
P (paired)	< 0.05	< 0.01
n	5	5

Values are means ± S.E. of n (number of tissues). pH_o represents bilateral pH across skin preparations. Tissues were perfused bilaterally with Ringer's solution B (Table 1; pH_o 8.0 or 6.4). During a stable impalement the apical solution was changed to Ringer's solution B + 0.33 mM 8-CPT-cAMP.

rized in Table 4 also shows that in 4 tissues in the presence of bilateral 75 mM K^+, cAMP induced depolarization of V_a was 4.0 ± 0.4 mV and an increase in I_{sc} was 5.5 ± 0.3 μA. These data indicate that under the experimental conditions in which cAMP-induced changes in V_a are significantly attenuated the cAMP-induced increase in I_{sc} is not blocked.

3.1.4. Effects of pH_o on cAMP action

In the next series of experiments we determined whether changes in pH_o affect cAMP-induced increase in I_{sc}. The effect of apical 0.33 mM 8-CPT-cAMP was investigated on I_{sc} and V_a at bilateral pH_o of either 8.0 or 6.4. Data in Table 5 shows that cAMP induced only very small increase in I_{sc} at pH_o 6.4. The average increase in I_{sc} was about 22%, but was not statistically significant. cAMP-induced changes in both V_a and I_{sc} were significantly greater at bilateral pH_o of 8.0 than at 6.4.

3.1.5. Effects pH_o in presence of cAMP

To study the effect of unilateral changes in pH_o in the continuous presence of cAMP, tissues were initially bathed bilaterally in frog Ringer's solution at pH 6.4 and treated with 0.33 mM apical cAMP. After the tissues had achieved new steady-state values of V_a and I_{sc} in the presence of cAMP, the apical solution was changed to Ringer's solution of pH 8.0, containing 0.33 mM cAMP. As summarized in Table 6, in 6 tissues pretreated with cAMP a change in apical solution pH from 6.4 to 8.0 depolarized V_a by 21 mV and increased I_{sc} by 29%. In an earlier study [29] qualitatively similar changes in V_a and I_{sc} were observed with unilateral changes in apical solution pH in the absence of cAMP. However, in the continuous presence of cAMP changing the pH of the basolateral solution to 8.0 did not produce any significant additional effects on either V_a or I_{sc} (data not shown).

Table 6
Effect of unilateral changes in apical pH_o in the presence of cAMP

Apical pH_o	V_a (mV)	I_{sc} (μA)
6.4	−43.4±7.2	13.2±2.8
8.0	−22.2±5.9	17.0±2.8
Δ	21.1±7.0	3.8±0.5 *
P (paired)	< 0.05	< 0.01

Values are means ± S.E. of $n = 6$. * 28.8±5.8% ($P < 0.01$). Tissues were perfused bilaterally with Ringer's solution B (Table 1; pH_o 6.4) and exposed to apical cAMP. After reaching a new steady-state in the presence of cAMP, the apical solution was unilaterally changed to Ringer's solution B (pH_o 8.0) containing, in addition, 0.33 mM 8-CPT-cAMP.

3.1.6. Effects of Cl^- substitution

We investigated the effect of unilateral Cl^- substitution on steady-state I_{sc}. Tissues were bathed in HCO_3^--free Cl^- Ringer's solution (Ringer's solution C; pH 8.0; Table 1) with ($n = 2$) or without ($n = 2$) 1 mM DIDS. Fig. 3 shows an experiment in presence of bilateral DIDS. At time zero, unilateral perfusion of Cl^--free solution (Ringer's solution D; Table 1) in the basolateral compartment resulted in a slow hyperpolarization of V_a from −79.5 to −85.7 mV ($\Delta V_a = -6.2$ mV) and a small decrease in I_{sc} from 10 to 7 μA ($\Delta I_{sc} = -3$ μA). However, the perfusion of Ringer's solution D on the apical side induced a rapid but transient depolarization of V_a from −85.7 mV to a maximum value of −67.4 mV ($\Delta V_a = 18.3$ mV) which was accompanied by a maximum increase in I_{sc} from 7 to 22 μA ($\Delta I_{sc} = 15$ μA). The initial depolarization of V_a and increase in I_{sc} demonstrated a strong near linear temporal relationship ($r = 0.99$; $n = 23$). A similar linear temporal relationship between V_a, I_{sc}, and pH_i was also observed with AVT and cAMP [30]. These rapid changes in V_a and I_{sc} were

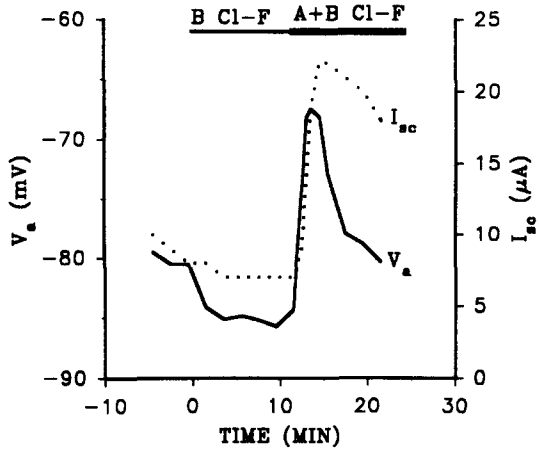

Fig. 3. Effect of Cl^- substitution. Solid thick line represents V_a and the dotted line represents I_{sc}. Thin horizontal bar on top of the figure (labelled as B Cl-F) represents the time period during which Cl^- was unilaterally substituted by NO_3^- in the basolateral (B) compartment. Thick horizontal bar (labelled as A + B Cl-F) represents the time period during which Cl^- was also substituted by NO_3^- in the apical compartment.

Table 7
Effect of Cl^- substitution

Apical/basolateral	V_a (mV)	I_{sc} (μA)	FR_a
Cl^-/Cl^-	−78.0±1.6	17.0±5.2	0.70±0.03
Cl^-/NO_3^-	−79.8±3.0	14.0±4.0	0.71±0.05
Δ_1	−1.8±1.4	−3.0±1.1	0.01±0.02
P (paired)	NS	NS	NS
Cl^-/Cl^-	−69.7±4.6	11.5±0.9	0.71±0.05
NO_3^-/Cl^-	−47.2±1.4	42.5±0.3	0.51±0.03
Δ_2	22.5±3.3	31.0±0.6	−0.20±0.04
P (paired)	< 0.025	< 0.001	< 0.05

Values are means ± S.E. of $n = 4$ in each group. Tissues were perfused bilaterally with Ringer's solution C (Table 1; pH_o 8.0) with ($n = 2$) or without ($n = 2$) 1 mM DIDS. During a successful impalement Ringer's solution C was unilaterally switched to Ringer's solution D (Table 1; pH 8.0).

followed by a slow partial recovery of both V_a and I_{sc}, to −81.3 mV and 17 μA. As summarized in Table 7, unilateral substitution of serosal Cl^- with NO_3^- in 4 tissues did not induce any significant changes in any of the parameters measured. However, in 4 additional tissues unilateral substitution of apical Cl^- by NO_3^- significantly depolarized V_a (by 22.5 mV) and increased I_{sc} (by 31 μA). These changes were accompanied by a significant decrease in FR_a.

3.2. Studies with double-barreled pH-sensitive microelectrodes

3.2.1. Effects of apical Cd^{2+} and Zn^{2+}

Divalent heavy metal ions (Cd^{2+} and Zn^{2+}) stimulate I_{sc} across frog skin epithelium by interfering with the Na^+-self-inhibition mechanism of the amiloride-sensitive apical Na^+ channels [22,42,43]. Therefore, we determined whether a divalent metal ion-induced increase in I_{sc} is accompanied by changes in V_a and/or pH_i. Tissues bathed in Ringer's solution C (Table 1; pH 7.4) were treated with either 2 mM Cd^{2+} ($n = 7$) or Zn^{2+} ($n = 6$) on the apical surface. Since both Cd^{2+} and Zn^{2+} gave qualitatively similar results the pooled data is summarized in Table 8. The Cd^{2+}- or Zn^{2+}-induced increase in I_{sc} was accompanied by depolarization of V_a and a decrease in FR_a. In the 10 tissues in which pH_i was measured with double-barreled pH-sensitive microelectrodes there was a significant increase in pH_i with metal ion treatment.

Table 8
Effect of apical Cd^{2+} or Zn^{2+}

Cd^{2+} or Zn^{2+}	V_a (mV)	I_{sc} (μA)	FR_a	pH_i
0	−74.6±3.2	21.7±3.0	0.66±0.04	7.428±0.114
2 mM	−59.6±3.4	34.6±3.8	0.59±0.03	7.505±0.119
Δ	15.0±2.5	12.9±1.8	−0.07±0.03	0.076±0.020
P (paired)	< 0.001	< 0.001	< 0.05	< 0.01
n	13	13	12	10

Values are means ± S.E. of n. Skin preparations were perfused with Ringer's solution C (Table 1; pH_o 7.4). During a successful impalement the apical solution was switched to Ringer's solution C containing, in addition, 2 mM Cd^{2+} ($n = 7$) or Zn^{2+}. ($n = 6$).

3.2.2. Effects of apical Cd^{2+} or Zn^{2+} on potential-induced changes in pH_i

Divalent heavy metal ions block H^+-conductive pathways (putative H^+ channels) in several cell types and tissues [6,20,27,31]. Therefore, we studied the effects of Cd^{2+} or Zn^{2+} on potential-induced changes in pH_i. In our earlier studies [29], when V_t was set at different values it induced changes in both V_a and pH_i in principal cells of frog skin. For step changes in V_t between −60 mV to 60 mV, there was a near linear relation between changes in V_a (ΔV_a) and pH_i (ΔpH_i). Therefore, we made several rapid step changes in V_t from 0 to 30 mV during a single stable impalement and measured the corresponding changes in V_a (ΔV_a) and pH_i (ΔpH_i). We used the ratio, $\Delta pH_i/\Delta V_a$ as a measure of potential-induced change in pH_i. The data with 2 mM apical Cd^{2+} is shown on Table 9. The ratio, $\Delta pH_i/\Delta V_a$ was not significantly affected by Cd^{2+}. Similar results were obtained with 2 mM Zn^{2+} (data not shown). Thus, Cd^{2+} and Zn^{2+} do not appear to block potential-induced changes in pH_i in principal cells.

3.2.3. Effects of EIPA or MIA

Bilateral exposure of skin preparations to either 10 μM EIPA or 1 μM MIA (blockers of the Na^+-H^+ exchanger) hyperpolarized V_a by 4.5 ± 0.8 mV, decreased I_{sc} by 17.0 ± 4.0 μA and decreased pH_i by 0.18 ± 0.04 units in 4 skin preparations. These data are consistent with the observation that a decrease in pH_o and/or pH_i is associated with a decrease in I_{sc}. In an earlier study from this laboratory [9], 10 μM EIPA completely blocked the Na^+-dependent recovery of pH_i in principal cells of frog skin.

Table 9
Effect of voltage clamping V_t in presence and absence of Cd^{2+}

	V_t (mV)	V_a (mV)	I_{sc} (μA)	pH_i	FR_a	$\Delta pH_i/\Delta V_a$
Control	0	−86.7 ± 0.9	15.0 ± 0.9	7.41 ± 0.09		
	30	−63.8 ± 0.7	−14.0 ± 1.1	7.73 ± 0.03		
Δ_1	30	22.9 ± 0.4	−29.0 ± 0.8	0.32 ± 0.06	0.76 ± 0.01	0.014 ± 0.001
Cd^{2+}	0	−57.8 ± 0.6	30.5 ± 2.5	7.52 ± 0.01		
	30	−40.8 ± 0.5	2.5 ± 0.5	7.73 ± 0.01		
Δ_2	30	17.0 ± 0.1	−28.0 ± 2.0	0.21 ± 0.02	0.57 ± 0.03	0.012 ± 0.001

Values are means ± S.E. of $n = 5$. All Δ_1 and Δ_2 values are significant at $P < 0.001$. Tissues were perfused with Ringer's solution C (Table 1; pH_o 7.4) with or without 2 mM Cd^{2+}.

4. Discussion

4.1. Role of Na^+-H^+ exchange, Cl^--HCO_3^- exchange and electrogenic Na^+-$(HCO_3^-)_n$ in cAMP action

Several studies suggest that ADH and/or cAMP induce an intracellular alkalinization [17,30,32,39]. A variety of hormones and agonists activate the Na^+-H^+ exchanger [13,33]. The activation of Na^+-H^+ exchanger has been demonstrated by vasopressin in A_6-Cl cell confluent monolayers [17], by aldosterone in target cells of the amphibian kidney [36] and in MDCK cells [35,48] and by insulin [33].

ADH acts via the cAMP second messenger pathway [19]. An increase in cAMP causes inhibition of Na^+-H^+ exchanger in cultured OK (Opossum kidney) epithelial cells [34], *Necturus* gallbladder epithelium [41], and small intestine [43]. However, Borgese et al. [3] have identified an isoform of Na^+-H^+ exchanger that is activated by cAMP. These differences in the response to cAMP in epithelial tissues may represent multiple isoforms of the Na^+-H^+ exchanger gene family on which cAMP has different effects [13]. cAMP also inhibits Cl^--HCO_3^- exchange in *Necturus* gallbladder epithelium [40]. Theoretically, both the activation of Na^+-H^+ and inhibition of Cl^--HCO_3^- exchangers could result in an intracellular alkalinization.

Data presented in Tables 2 and 3 demonstrate that cAMP-induced increase in I_{sc} is not blocked by complete removal of Cl^- and HCO_3^- or by the presence of DIDS, MIA or EIPA. These data suggest that Na^+-H^+ exchange, Cl^--HCO_3^- exchange, Cl^--OH^- exchange and electrogenic Na^+-$(HCO_3^-)_n$ are not involved in cAMP-induced increase in I_{sc}. In agreement with these data, in A_6-Cl cell monolayers, inhibition of the Na^+-H^+ exchanger did not prevent the stimulation of amiloride-sensitive I_{sc} by AVP [17]. In isolated frog skin preparations bathed in Cl^--free Ringer's solution (SO_4^{2-} replacing Cl^-) application of theophylline (a phosphodiesterase inhibitor) also significantly increased both the intracellular cAMP levels and I_{sc} [18]. The presence of Cl^- is, therefore, not essential to the effect of theophylline on Na^+ transport across frog skin.

4.2. Effects of unilateral Cl^- substitution

Biber et al. [2] have reported that unilateral replacement of Cl^- by methanesulfonate on either the mucosal or serosal side produced no immediate changes in I_{sc}, R_t or V_t of short-circuited whole skins from Southern variety of *Rana pipiens*. Replacement of Cl^- on the apical side alone induced a small transient hyperpolarization without any significant changes in I_{sc}, R_t or V_t, followed by a slow depolarization accompanied by a 70% decrease in I_{sc} and R_t. Sodium methanesulfonate substitution on the basolateral side did not cause immediate changes in V_a, I_{sc} or R_t and gave no transient hyperpolarization or depolarization. Based on similar results the Na^+-transporting principal cells of frog skin are generally considered to be nearly impermeable to Cl^- [2,7]. In the present study, unilateral replacement of Cl^- by NO_3^- in the basolateral side had no effect on V_a or I_{sc}. However, unlike the study by Biber et al. [2], in this study unilateral replacement of Cl^- by NO_3^- induced a rapid but transient depolarization of V_a and increase in I_{sc} (Fig. 3; Table 7). In a similar study on Northern variety of *Rana pipiens* [10] prompt responses in membrane potential were observed following replacement of external Cl^- on the serosal surface. In the *Xenopus laevis* distal nephron cell line A6-C1, both apical conductances for Na^+ and Cl^- are in the same cell. In these cells ADH induces a biphasic increase in I_{sc} [47]. However, we did not observe a biphasic effect of cAMP on I_{sc}.

In this study, replacing Cl^- with NO_3^- simultaneously induced both a depolarization of V_a and an increase in I_{sc} (Fig. 3; Table 7). These data indicate that changes in membrane potential result from an increase in Na^+ conductance of the apical cell membrane. As reviewed by Turnheim [46], channel-mediated apical Na^+ entry is not coupled to entry of Cl^-, but apical membrane Na^+ conductance is sensitive to the anionic composition of the solution bathing the luminal or outer surface of the epithelium. Gluconate reduces Na^+ transport in toad urinary bladder and frog skin from both sides by a decrease in cell volume, basolateral membrane K^+ conductance, and apical Na^+ entry. In this study Cl^- was replaced by NO_3^- while in other studies gluconate [10] or methanesulfonate [2] replaced Cl^-. Since impermeant anions like gluconate can significantly chelate extracellular Ca^{2+} [26], the differences in anion effects on transepithelial Na^+ transport could be partly explained by differences in extracellular or intracellular Ca^{2+} concentrations [19,46]. Secondly, NO_3^-, a permeant ion is not expected to affect the cell volume, basolateral K^+ conductance or volume regulatory mechanisms that are affected by non-permeant anions. Another possible way anion substitution can affect transepithelial Na^+ transport is by interfering with the Na^+-self-inhibition mechanism as has been observed with divalent metal ions [22,42,45]. However, at present the exact mechanisms for anion-dependent increase in I_{sc} is not known.

4.3. Effects of MIA and EIPA

In Northern variety of *Rana pipiens* application of 2 to 30 μM MIA decreased pH_i by about 0.18 pH units and in parallel experiments consistently increased I_{sc} and G_t, which were amiloride sensitive [5]. In our studies, MIA also decreased pH_i by about 0.18 pH units but this was accompanied by a decrease in I_{sc}. We have previously reported that there are other differences between these two varieties. In Northern frogs, Civan et al. [5] reported that both ADH and cAMP induce a decrease in pH_i and increase I_{sc}. However, we found that ADH- and cAMP-induced increase in I_{sc} was consistently associated with an

increase in pH_i in Southern frogs [30,32]. The experimental conditions and tissues were different, and different method (NMR) was used to measure pH_i, but the causes for the difference in pH_i response on I_{sc} is not known.

4.4. Role of potential-induced changes in pH_i in cAMP action

H^+ conductive pathways have been characterized in a variety of epithelial and non-epithelial cells and tissues [6,27,31]. In principal cells of frog skin the potential dependent step was localized to the apical cell membrane [29]. The potential-induced changes in pH_i are observed in the absence of Na^+, Cl^-, and HCO_3^-. This indicates that potential-induced changes in pH_i can occur in the absence of Na^+ transport, Na^+-H^+ exchange, Cl^--HCO_3^- exchange and electrogenic Na^+-$(HCO_3^-)_n$ cotransport [8,28,29].

Divalent heavy metal ions Cd^{2+} and Zn^{2+} (Tables 8 and 9) which activate the apical Na^+ channels depolarize V_a and increase pH_i. In addition, under the conditions in which Cl^--HCO_3^- and Cl^--OH^- exchangers are not active (Table 7), the manipulation of V_a by unilaterally replacing apical Cl^- was accompanied by an increase in I_{sc}. Although no pH_i measurements were made in these experiments, we suggest a role for potential-induced changes in pH_i in modulating I_{sc}.

In our earlier study [30] we observed that: (i) ADH- and cAMP-induced increase in pH_i proceeded towards the pH_{eq} value calculated from the Nernst equation. This suggests that changes in pH_i could be simply due to a passive mechanism. (ii) There was consistently a close relationship between the changes in V_a and in pH_i initiated by ADH and cAMP. (iii) In the presence of AVT voltage clamping V_a at different levels gave a nearly linear relationship between ΔV_a and ΔpH_i. These data suggested that there is a voltage-dependent shift in pH_i after ADH and cAMP treatment that is temporally related to an increase in I_{sc}.

4.5. H^+ leak via apical Na^+ channels

In several cell types and tissues divalent heavy metal ions block putative H^+ channels [6,27,32]. However, in our studies neither Cd^{2+} or Zn^{2+} had effects on the potential-induced changes in pH_i (Table 9) but served as activators of apical Na^+ channels. In our previous studies, apical Na^+ channel blockers, amiloride and benzamil, inhibited potential-induced changes in pH_i [29]. Taken together these data suggest that Na^+ channels are involved in potential-induced changes in pH_i, presumably by leakage of H^+ across the channel [28,29,31]. Most direct evidence for H^+ leak via the apical Na^+ channels has been demonstrated in patch-clamp studies on hamster taste cells [16].

4.6. Role of pH_i in cAMP action

Several experiments suggest that changes in pH_i modulate cAMP action. First, a strong temporal relationship is observed between cAMP-induced increase in pH_i and I_{sc} [30]. Second, a decrease in apical solution pH hyperpolarized V_a and decreased I_{sc} in the presence of cAMP (Table 6). Third, a decrease in apical solution pH from 8.0 to 6.4 decreased cAMP-induced effects on V_a and I_{sc}. Since the pH-sensitivity of apical Na^+ channels has been reported in patch-clamp studies [38], it is suggested that changes in pH_o and/or pH_i modulate cAMP action indirectly by effects on apical Na^+ channels.

Two sets of observations indicate that changes in pH_i do not play a strict regulatory role but are only permissive in the cAMP regulation of apical Na^+ conductive transport in frog skin epithelium. First, when voltage-induced changes in V_a were attenuated by treatment with high K^+ (Figs. 1 and 2; Table 4) the cAMP-induced increase in I_{sc} was not blocked. We have shown that under a variety of experimental conditions, changes in V_a (ΔV_a) and pH_i (ΔpH_i) demonstrate a near linear temporal relationship in principal cells of frog skin [28–30]. These data suggest that in presence of basolateral high K^+, progressive attenuation of cAMP-induced depolarization of V_a was accompanied by a parallel decrease in potential-induced changes in pH_i. In the presence of 75 mM basolateral K^+, the mean cAMP-induced depolarization of V_a was only 4 mV, however, under these conditions I_{sc} increased from a mean value of 11.5 to 17.0 μA (an increase of 47.8%; Table 4). A change in V_a of 4 mV will contribute a potential-induced increase in pH_i of only 0.02 pH units [8]. Such a small change in pH_i cannot account for the observed increase in I_{sc}. Second, in the presence of cAMP an increase in pH_o and/or pH_i (Table 6) produced an additional increase in I_{sc}. From these data it may be inferred that changes in pH_i are not an essential requirement for the cAMP action [33]. Although changes in external pH can modulate ADH-induced increase in endogenous cAMP [23], these data indicate that when intracellular cAMP is increased by exogenous cAMP, its effects can be modulated independently by pH.

The role of potential-induced changes in pH_i in renal epithelial cells is not clear. Ouabain [44], amiloride and benzamil [14], drugs known to affect cell membrane potentials, did not have any effect on pH_i in rat renal cortical collecting tubules. Manipulation of mineralocorticoid status in vivo using low-Na^+ diet or the diuretic furosemide induced no changes in pH_i of rat cortical collecting tubules [37]. These data indicate that potential-induced changes in pH_i may be restricted to certain cell types and may not occur with chronic hormone exposure.

During ADH and/or cAMP action a primary increase in apical Na^+ conductance could cause the increase in pH_i via changes in cell potential [30]. Since the activity of apical Na^+ channels is increased by an increase in pH_i

[38], cell alkalinization would tend to augment the direct response to ADH. It needs to be stressed that ADH- and/or cAMP-induced changes in I_{sc}, V_a and pH_i occurred simultaneously within the time resolution given by the microelectrode technique [30]. It is, therefore, not possible to establish an order of succession for these events from microelectrode studies. Perhaps this issue can be resolved by monitoring changes in pH_i by more sensitive methods with a faster time resolution (e.g., by pH-sensitive intracellular dyes) simultaneously with changes in I_{sc}. Further studies are needed to measure ADH- and/or cAMP-induced temporal relationships between pH_i and I_{sc} [30] in principal cells under the conditions in which one or more pH regulatory mechanisms are either activated or inhibited.

In conclusion, data presented in this paper demonstrate that changes in pH_o and/or pH_i modulate the cAMP effects on overall Na^+ transport across frog skin epithelium. The cAMP-induced increase in I_{sc} does not involve the participation of Na^+-H^+ exchanger, Cl^--HCO_3^- exchanger or the electrogenic Na^+-$(HCO_3^-)_n$ cotransport. The observations that unilateral apical Cl^- substitution induce depolarization of V_a and increase in I_{sc}, and divalent metal ions depolarized V_a and increase both pH_i and I_{sc}, support, but do not prove that cAMP-induced changes in pH_i observed in this and previous study are due to the potential-induced changes in pH_i. Since cAMP was able to induce an increase in I_{sc} in experiments in which depolarization of V_a was greatly attenuated by the presence of basolateral high K^+, and changes in pH_o and/or pH_i can independently modulate cAMP action, these data indirectly suggest that a change in pH_i is not an essential requirement for cAMP action. Thus changes in pH_i are only permissive in the cAMP-induced effects on I_{sc}. This is unlike the second messengers, cAMP and $[Ca^{2+}]_i$, which play a strict regulatory role during hormone action [33].

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