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Osmotic Regulation of Na^+ Transport Across A6 Epithelium: Interactions with Prostaglandin E_2 and Cyclic AMP

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Abstract. Previous work from this laboratory has shown that apical membrane sodium channel activity is stimulated by serosal hyposmotic solutions (Wills, Millinoff & Crowe, 1991). In the present study, we determined whether this stimulation of sodium transport is additive with the actions of prostaglandin E_2 (PGE_2) or cyclic AMP (cAMP).

Addition of exogenous PGE_2 (100 nM; serosal bath) to isosmotic solutions led to large increases in the amiloride-sensitive short-circuit current (I_{sc}) and transepithelial conductance (G_t), whereas no significant effects of PGE_2 were observed in hyposmotic serosal solutions. Subsequent addition of mucosal amiloride reduced I_{sc} by ~95% and G_t by ~60%. Inhibition of endogenous PGE_2 production by blockers of phospholipase A_2 activity (quinacrine or 3[4-octadecyl]-benzoylacrylic acid; OBBA), or inhibition of cyclooxygenase activity by indomethacin reduced the stimulation of I_{sc} and G_t by hyposmotic solutions. Addition of forskolin (FSK) or 3-Isobutyl-1-methylxanthine (IBMX) also resulted in approximately twofold increases in the amiloride-sensitive I_{sc} and G_t and abolished the effects of subsequent hyposmotic challenge. The effects of forskolin, PGE_2, and hyposmotic challenge were diminished by pretreatment with H89, a protein kinase A (PKA) inhibitor. We conclude that osmotic regulation of sodium channel activity interacts with multiple intracellular signaling pathways, specifically the arachidonic acid metabolic pathway and the cAMP/PKA intracellular messenger cascade.

Key words: Amphibian renal cell line — Arachidonic acid — cAMP — Cell volume — Epithelia — Phospholipase A_2

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Introduction

Many ion transport proteins show altered activity in cells exposed to hypotonic or hypertonic solutions (*c.f.* Strange, 1994). Previous work from this laboratory has shown that solution osmolality has a potent effect on sodium absorption across cultured amphibian renal A6 epithelia cells (Wills et al., 1991; Crowe et al., 1995). Specifically, small (15%) reductions in serosal solution osmolality produced a nearly twofold increase in the number of conducting sodium channels, whereas hyperosmotic solutions decreased sodium channel activity. The effects of hyposmotic solutions were similar to the effects of hyposmotic solutions on transepithelial sodium currents previously reported for frog skin (MacRobbie & Ussing, 1961) and toad urinary bladder (Lipton, 1972). At present, little is known about the intracellular signaling events that lead to these changes in sodium channel activity.

Hyposmotic solutions are known to increase intracellular cAMP in some cell types (e.g., lymphoma cells; Watson, 1990) and cAMP is known to stimulate amiloride-sensitive sodium transport in amphibian tight epithelia and A6 cells (*c.f.* Frazier & Yorio, 1992; Perkins & Handler, 1981; Marunaka & Eaton 1990). Prostaglandins are also potent stimulators of sodium transport in amphibian tight epithelia (Els & Helman, 1981, 1997; Hall et al., 1976). Although less is known about the role of prostaglandins in cellular responses to hyposmotic challenge, prostaglandins E_1 and E_2 are known to elevate intracellular cAMP levels in amphibian tight epithelia (Hall et al., 1976; Pohlman et al., 1983) and A6 cells (Yanase & Handler, 1986; Nisato & Marunaka, 1997).

In the present study, we tested the hypothesis that

hyposmotic stimulation of amiloride-sensitive sodium currents in A6 cells involves the actions of cAMP and/or PGE_2. Specifically, we compared the effects of addition of exogenous PGE_2 or forskolin in isosmotic and hyposmotic solutions and determined the effects of prostaglandin synthesis inhibitors and a blocker of protein kinase A (PKA). The results suggest that arachidonic acid, prostaglandin E_2 cAMP, and PKA play a role in the stimulation of amiloride-sensitive sodium channels by hyposmotic solutions. In addition, PGE_2 and cAMP stimulate an amiloride-insensitive conductance not activated by hyposmotic challenge. A portion of this work has appeared in abstract form (Matsumoto & Wills, 1994).

Materials and Methods

Cell Culture

The methods used in this paper are similar to those of Wills et al. (1991). Briefly, A6 cells (American Type Culture Collection; Rockville, MD) between passages 70–80 were grown on permeable Anocell™ filters (Whatman; Clifton, NJ) for 2–4 weeks. The cells were fed three times per week with Dulbecco's Modified Eagle's Medium (Gibco Laboratories; cat. #84-5022; Grand Island, NY) supplemented with penicillin/streptomycin (Gibco) and 10% fetal bovine serum (Hyclone Laboratories; Logan, UT). The osmolality of the medium was 200 mosmol/kg water. The cells were kept in an incubator at 27°C and 1% CO_2 in air.

Solutions

A6 monolayers were bathed in culture medium that did not contain serum or phenol red. In some experiments, a modified Ringers solution was used, which contained (in mM): 74.4 NaCl, 5.4 KCl, 8 $NaHCO_3$, 1.4 $CaCl_2$, 1.7 $MgSO_4$, 0.9 NaH_2PO_4, 5.5 glucose, 1 Na pyruvate, and 1 HEPES. Isosmotic solutions were defined as 200 mosmol/kg H_2O; solution osmolality was adjusted by the addition of mannitol. The Ringer's solution and the culture medium produced similar results, therefore data for these solutions were combined.

Electrical Measurements

A6 monolayers were put into a water-jacketed (28°C) Ussing chamber and bubbled with 1% CO_2 and stirred with magnetic stir bars. The tissue was initially bathed in symmetric isosmotic solution (200 mosmol/kg H_2O). The serosal osmolality was then reduced by replacing the serosal bath with a hyposmotic (mannitol-free) solution (170 mosmol/kg H_2O). The solution change was complete within two minutes.

Transepithelial electrical measurements were made under open-circuit conditions with Ag/AgCl electrodes connected to an automatic voltage clamp under computer control. The transepithelial potential (V_t) was continuously monitored, except to determine the transepithelial conductance (G_t) and short circuit current (I_{sc}). G_t was calculated as the ratio of the change in current to voltage ($G_t = \Delta I/\Delta V$). I_{sc} was calculated as $G_t \times V_t$. Both I_{sc} and G_t were normalized to epithelial area (4.2 cm^2).

Chemicals

3[4-octadecyl]-benzoacrylic acid (OBAA), H89, 3-isobutyl-1-methylxanthine (IBMX), were from Biomol (Plymouth Meeting, PA). Prostaglandins and forskolin were from Calbiochem (San Diego, CA) and quinacrine 2HCl was from ICN (Costa Mesa, CA). Indomethacin was from Sigma (St. Louis, MO). Quinacrine was dissolved in water and PGE_2, was dissolved in ethanol. All other drugs were dissolved in DMSO.

Statistics

The data presented are means ± SEM. Data were analyzed using either a one-way analysis of variance (ANOVA) and conservative post-tests, or paired *t*-tests as appropriate. Statistical significance was defined as $P < 0.05$.

Results

Stimulation of Transepithelial Na^+ Transport by Hyposmotic Serosal Solutions

Figure 1 illustrates the effects of reducing the osmolality of the serosal bathing solution from 200 mosmol/kg H_2O to 170 mosmol/kg H_2O (HYPO). In agreement with our previous results which demonstrated increased sodium transport and sodium channel activity (Wills et al., 1991; Crowe et al., 1995), increases in I_{sc} and G_t were detectable within 2–5 min and reached a new plateau level within 30 min. I_{sc} and G_t were both significantly increased from 16 ± 5 $\mu A/cm^2$ and 368 ± 58 $\mu S/cm^2$, respectively, to 37 ± 3 $\mu A/cm^2$ and 546 ± 47 $\mu S/cm^2$ ($P < 0.02$ and $P < 0.05$, respectively; $n = 5$). Addition of amiloride (50 μM) to the apical bath of HYPO tissues, essentially abolished the I_{sc} (1 ± 1 $\mu A/cm^2$) and significantly reduced G_t to 202 ± 24 $\mu S/cm^2$ ($P < 0.05$). Pretreatment with amiloride prevented the stimulation of I_{sc} and G_t (*data not shown*) by serosal hyposmotic solutions. In the following experiments, we assessed the interactions of this hyposmotic stimulation of transepithelial Na^+ transport with the effects of prostaglandin E_2 or elevated intracellular cAMP. Data for hyposmotic solutions are reported for the 30 min time period, i.e., for steady-state levels of I_{sc} and G_t.

Effects of Prostaglandin E_2 (PGE_2)

As discussed above, exogenous prostaglandins are known to increase Na^+ transport across amphibian native tight epithelia such as the toad urinary bladder and frog skin (*c.f.*, Frazier & Yorio, 1992; Els & Helman 1981, 1997). This capacity for Na^+ transport stimulation is retained in A6 cells as shown in Fig. 2. Addition of PGE_2 (100 nM) to the serosal solution significantly increased I_{sc} (from 25 ± 5 to 43 ± 5 $\mu A/cm^2$, $P < 0.05$) and G_t

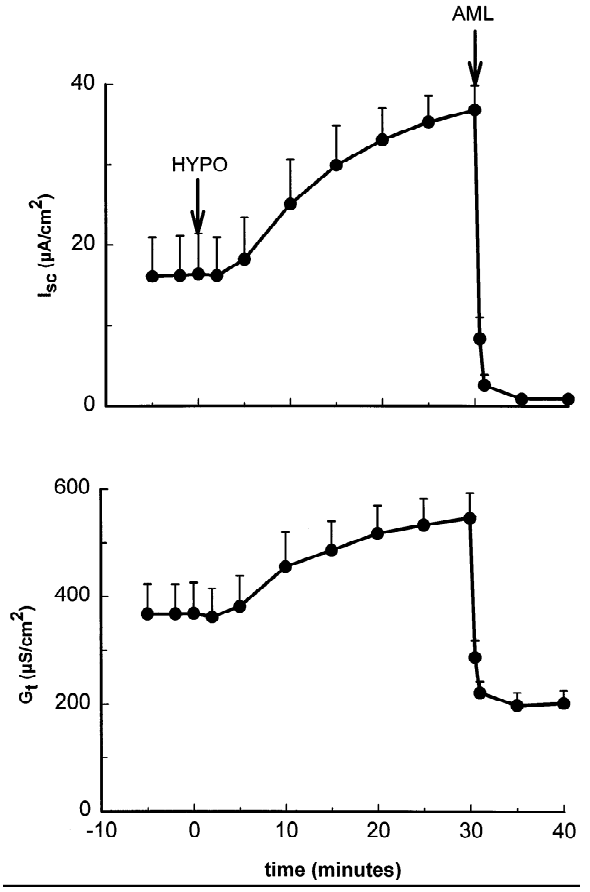

Fig. 1. Time course of the effects of a hyposmotic serosal bath on I_{sc} and G_t. At time = 0, the bath osmolality was changed from an isosmotic to hyposmotic solution (HYPO). The change in osmolality was done after the baseline I_{sc} and G_t became stable (~30–60 min) after mounting the epithelium in the Ussing chamber). After 30 min, 50 μM amiloride was added into the apical bath ($n = 5$).

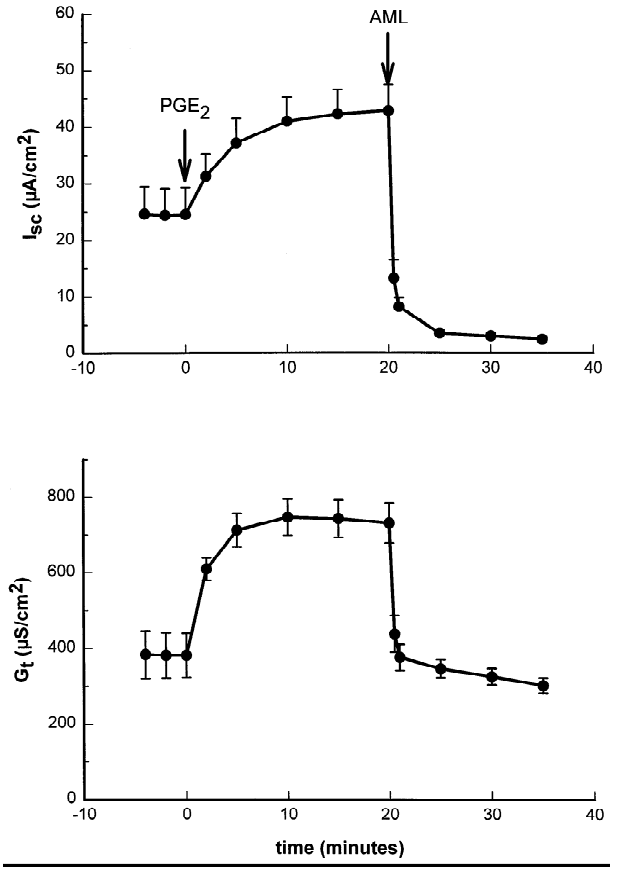

Fig. 2. The effects of PGE_2 on I_{sc} and G_t under isosmotic conditions. At time = 0, 100 nM PGE_2 was added to the serosal bath. After 20 min, 50 μM apical amiloride was added. $n = 5$.

(from 381 ± 59 to 731 ± 53 μS/cm^2, $P < 0.001$) within 10 min ($n = 5$). Figure 3 shows the early time points for the same data as Fig. 2, now shown as individual experiments. Note that in all cases, a gradual stimulation of I_{sc} and G_t were observed. Addition of mucosal amiloride (50 μM) to PGE_2-treated tissues significantly decreased G_t and I_{sc} (*see* Fig. 2).

Because the actions of PGE_2 and serosal hyposmotic solutions were similar, we next investigated the interaction between PGE_2 and hyposmotic serosal solutions. Figure 4 presents a typical experiment in which the epithelium was subjected to serosal hyposmotic challenge following PGE_2 treatment. In contrast to the usual effects of HYPO (*see above*), exposure of PGE_2-treated tissues to hyposmotic serosal solutions led to an unexpected, small transient decrease in I_{sc} (−8 ± 4%; $n = 4$), and a slow decline in G_t (−22 ± 5% over 30 min; $n = 4$). The transient effects may reflect chloride currents as they were not observed when the same experiments were performed following chloride replacement in both bathing solutions by cyclamate or gluconate. These results indicate that the effects of hyposmotic solutions and PGE_2 on Na^+ transport are not additive.

In another set of experiments, PGE_2 was added under HYPO conditions (i.e., hyposmotic serosal solution) following stabilization of the transepithelial electrical parameters. Under these conditions, there was a significantly smaller increase in I_{sc} ($\Delta I_{sc} = 12.1 \pm 0.8$ μA/cm^2 for HYPO compared to $\Delta I_{sc} = 18.5 \pm 1.6$ μA/cm^2 for isosmotic solutions: $P < 0.05$). Similarly, the stimulation of G_t by PGE_2 was also significantly smaller in hyposmotic solutions ($\Delta G_t = 250 \pm 12$ μS/cm^2 for HYPO compared to $\Delta G_t = 374 \pm 21$ μS/cm^2 for isosmotic solutions; $P < 0.05$). Consequently, osmotic and prostaglandin regulatory mechanisms do not act independently.

Some differences between the actions of PGE_2 and hyposmotic solution were also observed. First, a small amiloride-insensitive current was observed in PGE_2-treated tissues ($I_{sc} = 2.5 \pm 0.5$ μA/cm^2, $n = 5$, $P < 0.05$ compared to zero), but not in cells exposed to serosal hyposmotic solutions (compare Figs. 1 and 2). Consis-

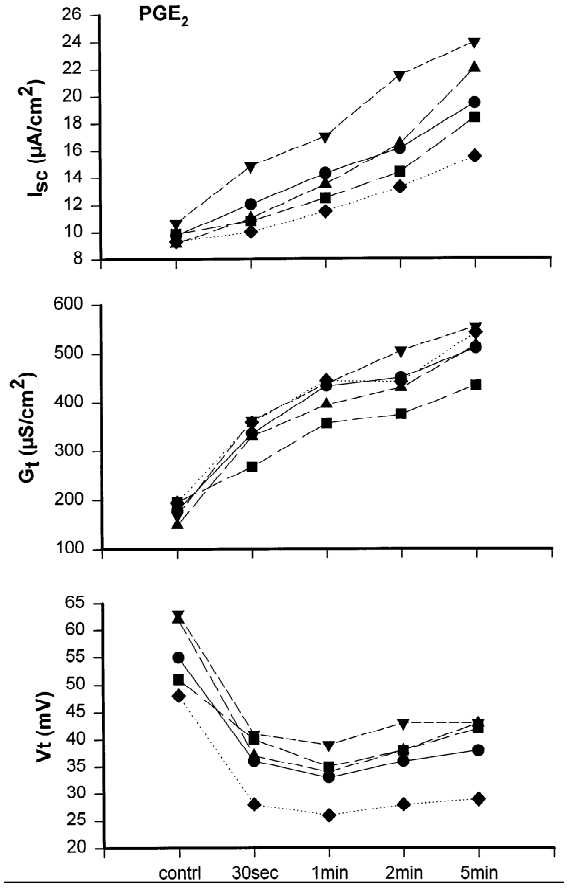

Fig. 3. Short latency effects of PGE_2. Transepithelial voltage (V_T) and I_{sc} and G_t data are from Fig. 2 and show results for individual experiments.

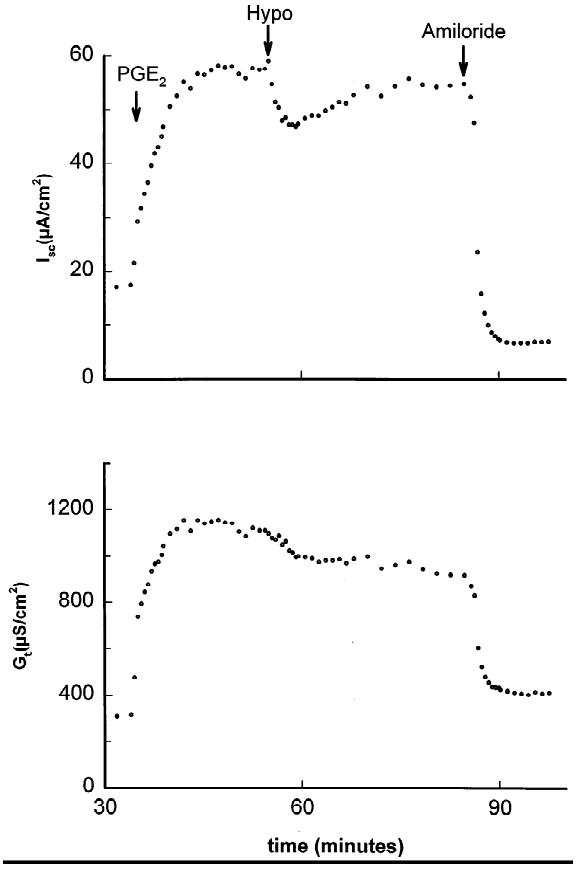

Fig. 4. Typical experiment showing the effects of serosal hyposmotic challenge on an epithelium that was previously treated with PGE_2 (100 nM serosa).

tent with these findings, in preliminary experiments, we observed that addition of 100 nM PGE_2 to amiloride-treated tissues led to a small increase in I_{sc} (~5 microamps) and increased G_T (~250 μS/cm^2). Amiloride-treated tissues did not respond to hyposmotic solutions. Finally, in contrast to the effects of hyposmotic solutions (Wills et al., 1991), PGE_2 decreased V_T (Fig. 3). These results suggest that PGE_2 activates other conductance(s) in addition to the apical membrane sodium conductance. The actions of PGE_2 were apparently unique since other prostaglandins, specifically $PGF_{2\alpha}$ or carbacyclin, a PGI_2 agonist, did not significantly affect I_{sc} or G_t (*data not shown*).

Effects of Pharmacological Blockers of PGE_2 Production

As noted in Fig. 5, the enzyme phospholipase A_2 (PLA_2) and cyclooxygenase are responsible for the generation of prostaglandin E_2 production (*c.f.* Frazier & Yorio, 1992). In the following experiments, we examined the effects of two classes of pharmacological inhibitors, specifically: (i) indomethacin, a cyclooxygenase inhibitor (Mizuno, Yamamoto & Lands, 1982), and (ii) two blockers of phospholipase A_2 activity, quinacrine (Lapetina, 1982) and 3[4-octadecyl]-benzoylacrylic acid (OBAA; Kohler et al., 1992).

Treatment of the epithelia for 15–30 min with 10 μM indomethacin under isosmotic conditions significantly decreased I_{sc} from 18 ± 3 μA/cm^2 to 6 ± 2 μA/cm^2 and G_t was reduced from 387 ± 27 to 263 ± 28 μS/cm^2 (n = 5; *see* Fig. 6). Indomethacin also significantly reduced the stimulatory effects of hyposmotic solutions on I_{sc} and G_T, i.e., I_{sc} was ~40% and G_t was ~60% of values for untreated cells (*see* Fig. 6). Consequently, blockage of cyclooxygenase activity reduced the basal rate of Na^+ transport and its enhancement by hyposmolality.

Similar inhibitory effects on basal I_{sc} and G_t were found for PLA_2 inhibitors. As summarized in Table 1, exposure of the cells to OBAA (10 μM; n = 4) for one hour or quinacrine (50 μM, n = 3; QUIN) for 45 min significantly reduced I_{sc} and G_T. I_{sc} decreased by 71 ± 43% for OBAA and by 85 ± 10% for QUIN ($P < 0.05$ for

both drugs). Basal G_t values were decreased by 42 ± 6% for OBAA and by 45 ± 5% for QUIN ($P < 0.05$ for both drugs). The effects of hyposmotic challenge on I_{sc} and G_t were also greatly reduced in the presence of OBAA or QUIN (*see* Table 1). These findings are consistent with a role for PLA_2 and prostaglandin in the maintenance of basal sodium transport rates and in facilitating stimulation of this process by hyposmotic solutions.

Effects of Adenyl Cyclase/cAMP

In the next series of experiments, we assessed the interactions of hyposmotic stimulation of the amiloride-sensitive transport with cAMP mediated stimulation of I_s and G_T by serosal addition of forskolin (FSK) an activator of adenyl cyclase (Uneyama, Uneyama & Akaike, 1993) or 3-isobutyl-1-methlxanthine (IBMX), a nonspecific inhibitor of phosphodiesterases (Beavo et al., 1971).

Forskolin

As shown in Fig. 8, following addition of 10 μM FSK, I_{sc} and G_t significantly increased within 5 min from 15.1 ± 1.4 μA/cm^2 and 316 ± 9 μS/cm^2 to 31.5 ± 3.2 μA/cm^2 and 741 ± 34 μS/cm^2. Preliminary dose-response experiments indicated that this dosage of FSK was maximally effective. As in the case of HYPO and PGE_2, subsequent addition of mucosal amiloride decreased I_{sc} and G_t (I_{sc} = 1 ± 1 μA/cm^2; G_t = 344 ± 22 μS/cm^2; $P < 0.05$ for both; $n = 4$).

Similar to the effects of PGE_2, prior treatment of the epithelia with 10 μM FSK abolished the effects of hyposmotic challenge on G_t and I_{sc}, i.e., I_{sc} was not significantly altered by changing from an isosmotic to a hyposmotic serosal solution (46.2 ± 1.4 to 47.8 ± 1.5 μA/cm^2, $n = 5$). The corresponding G_t values were also not significantly changed, averaging 883 ± 68 and 783 ± 51 μS/cm^2, for isosmotic and hyposmotic solutions, respectively.

In additional experiments, forskolin was added to isosmotic or hyposmotic serosal solutions. As shown in Fig. 9, the maximal I_{sc} or G_t stimulated by forskolin did not significantly differ between cells bathed in hyposmotic or isosmotic solutions (I_{sc} = 46.5 ± 1.5 and 41.8 ± 3.7 μA/cm^2 and G_t = 895 ± 68 and 770 ± 90 μS/cm^2, respectively). Therefore, forskolin and hyposmotic stimulation of sodium transport were not additive, similar to the lack of additive effects observed for PGE_2.

IBMX

Intracellular levels of cAMP in A6 cells can be increased by blocking degradation by phosphodiesterase (Nisato & Marunaka, 1997; *see* Fig. 7). We compared the effects of the phosphodiesterase inhibitor IBMX on transepithelial electrical parameters using three different experimental protocols: (I) IBMX (100 μM) treatment in symmetrical isosmotic solutions, (II) IBMX addition to a hyposmotic serosal solution, and (III) IBMX (100 μM) addition to symmetrical isosmotic solutions followed by replacement of the serosal bath with hyposmotic solution.

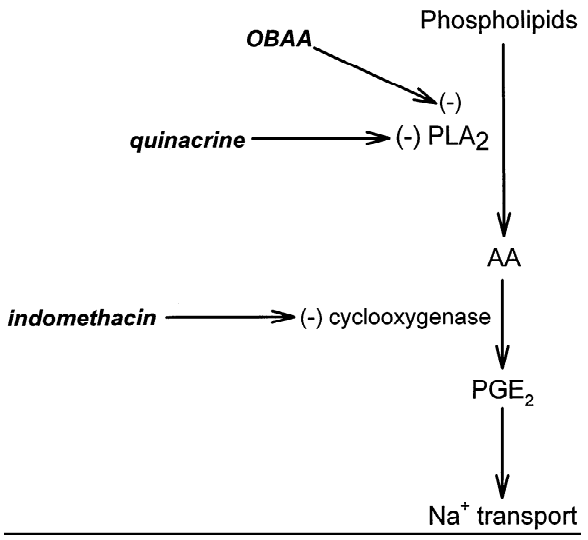

Fig. 5. Overview of phospholipase A_2 (PLA_2) generation of arachidonic acid (AA) and subsequent prostaglandin synthesis in renal cells. The products of cyclooxygenase activity are further processed by peoxidases and synthases to produce prostaglandin E_2 (PGE_2). Pharmacological blockers indicated for this pathway include 3(4-octadecyl)-benzoylacrylic acid (OBAA), quinacrine, and indomethacin.

Addition of IBMX to isosmotic solutions resulted in large increases in I_{sc} and G_t (54 ± 14% and 84 ± 18%, respectively; $n = 6$) whereas smaller increases were obtained in hyposmotic solutions (ΔI_{sc} = 14 ± 4% and ΔG_t = 41 ± 14%; Protocol II.; $n = 3$). As indicated in Table 2, the increases in I_{sc} and G_t induced by IBMX were reduced following the addition of amiloride (50 μM) to the mucosal solution. More importantly, exposure to IBMX abolished the large stimulation of I_{sc} and G_t usually evoked by hyposmotic solutions (Protocol III.; ΔI_{sc} = −17 ± 3% and ΔG_t = 5 ± 4%; NS). These findings provide further evidence that hyposmotic stimulation of sodium transport interacts with the cAMP pathway.

Inhibition of PKA Action

Elevations of cyclic AMP lead to activation of protein kinase A (PKA) which may activate sodium transport. To further test the hypothesis of cAMP involvement in the hyposmotic activation of I_{sc}, we examined the effects of H89, a PKA inhibitor (Chijiwa et al., 1990). Table 3 compares transepithelial electrical parameters for tissues with or without exposure to 10 μM H89 (added to the

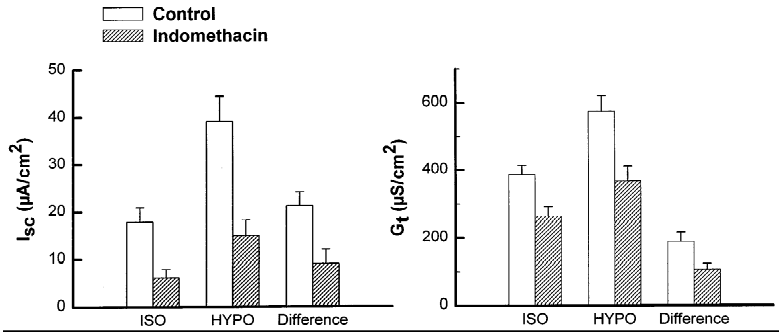

Fig. 6. The effect of indomethacin (10 μM serosa) on the short-circuit current (I_{sc}) and transepithelial conductance (G_t) in isosmotic (ISO) and hyposmotic (HYPO) serosal bathing solutions (n = 5).

Table 1. Effects of PLA_2 inhibitors serosal bathing solution

	I_{sc} (μA/cm²)	G_t (μS/cm²)
Control		
Isosmotic	20 ± 4	403 ± 49
Hyposmotic	39 ± 2**	562 ± 47**
Difference N = 5	19 ± 2	159 ± 33
OBAA[a]		
Isosmotic	6 ± 3*	233 ± 13*
Hyposmotic	15 ± 4*	321 ± 20*,**
Difference N = 4	9 ± 2*	89 ± 12*
Quinacrine[b]		
Isosmotic	3 ± 0.3*	220 ± 11*
Hyposmotic	6 ± 0.4*,**	268 ± 12*,**
Difference N = 3	3 ± 0.2*	48 ± 4*

[a] 10 μM, 60 min, serosal bath; n = 4; [b] 50 μM, 45 minutes, serosal solution; n = 3
* $P < 0.05$ compared to control; **$P < 0.05$ compared to isosmotic

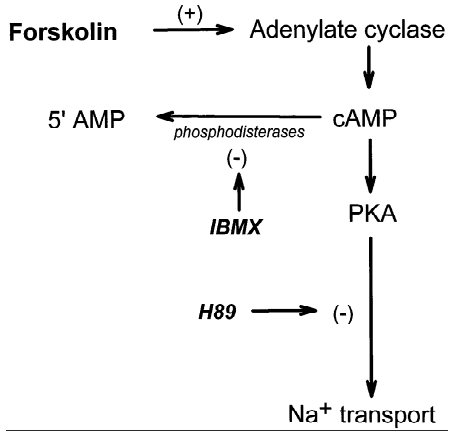

Fig. 7. Stimulators and pharmacological blockers of intracellular cAMP generation. PKA is cAMP-dependent protein kinase and H89 is n[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide.

serosal solution for 40–60 min). In isosmotic solutions, H89 treated epithelia had baseline I_{sc} values that were 87 ± 18% lower and baseline G_t values that were 33 ± 13% lower than untreated paired controls ($P < 0.05$). The increases in these parameters evoked by hyposmotic serosal solutions were approximately 15 and 35% of the ΔI_{sc} and ΔG_t values in untreated cells, respectively.

Pretreatment with H89 also inhibited the effects of PGE_2. Following addition of 100 nM PGE_2 to the serosal bath I_{sc} was 6 ± 2 μA/cm² for H89-treated tissues and 55 ± 2 μA/cm² for untreated controls. G_T values were 326 ± 38 μS/cm² and 733 ± 56 μS/cm², respectively.

Comparison of the effects of PGE_2, Forskolin, and Hyposmotic solutions

Figure 10 summarizes the effects of PGE_2, FSK, and hyposmotic challenge as a percentage of the control current and conductance values in isosmotic bathing solutions. Comparison of these data reveals that PGE_2 stimulation was less effective than FSK or Hypo in stimulating I_{sc}, in agreement with studies of sodium channel activation (Kokko et al., 1994). In contrast, FSK and HYPO stimulation resulted in comparable increases in I_{sc}, although the effects of HYPO were slower.

With respect to G_t, the relative effects of PGE_2, FSK, and HYPO showed a different pattern. Specifically, HYPO showed the least change in G_t, PGE_2 resulted in moderate increases and FSK was the most effective in increasing G_t. Given the results for I_{sc}, these findings suggest that FSK and PGE_2 activate an additional conductance not activated by HYPO.

Effects in Hyperosmotic Solutions

Previous studies from this laboratory (Wills et al., 1991) have reported that sodium channel activity is inhibited during exposure to hyperosmotic serosal solutions. We assessed whether this inhibition could be reversed by forskolin.

As shown in Table 4, replacement of the serosal bathing solution with a hyperosmotic solution (containing 120 mM mannitol; osmolality = 290 mosmol/kg H_2O) essentially abolished I_{sc} and significantly decreased G_T. Under these conditions, mucosal amiloride

was ineffective (I_{sc} = 1 ± 0.1 μA/cm^2 and G_t = 359 ± 57 μS/cm^2, n = 4; NS) in agreement with our previous reports (Wills et al., 1991). As also indicated in Table 4, in tissues treated with forskolin, hyperosmotic serosal solutions had a similar effect. Under these conditions mucosal amiloride significantly decreased I_{sc} by 4 ± 1.4 μA/cm^2 and G_t by 60 ± 2 μS/cm^2 (n = 4). Consequently the effects of FSK on the amiloride-sensitive current and conductance pathway were decreased in hyperosmotic solutions compared to isosmotic solutions (ΔI_{sc} ~30 μA/cm^2 and ΔG_t ~400 μS/cm^2, *see* Fig. 8 above). In two experiments, FSK was added to tissues bathed in serosal hypersosmotic solutions. I_{sc} increased from 0.8 ± 0.5 μA/cm^2 to 3 ± 0.5 μA/cm^2 and G_t increased from 209 ± 23 μS/cm^2 to 414 ± 140 μS/cm^2. Consequently, forskolin was not able to overcome the inhibitory effects of hyperosmotic solutions (compare data in Fig. 8 and Table 4). Preliminary measurements with PGE_2 yielded similar results.

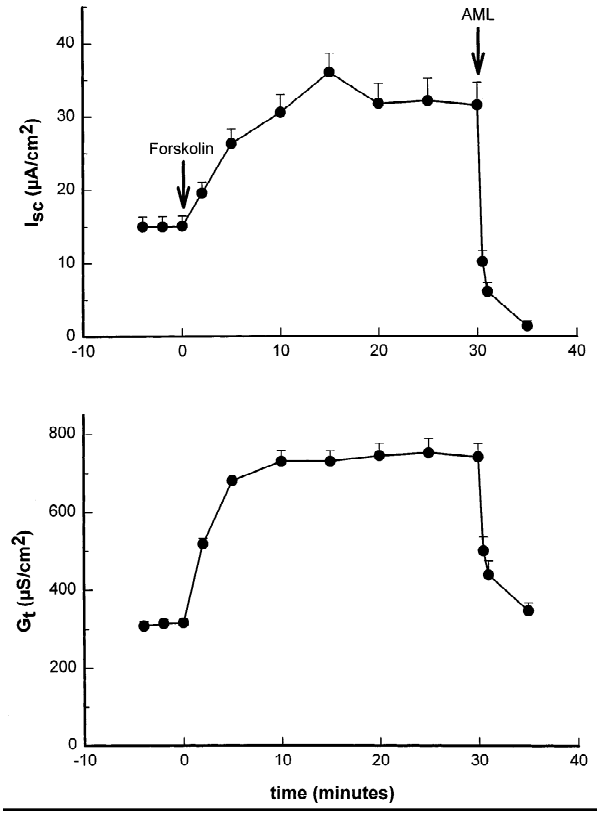

Fig. 8. The effect of forskolin on I_{sc} and G_t in an isosmotic bath. At time = 0, 10 μM serosal forskolin was added. AML indicates addition of 50 μM amiloride to the mucosal solution (n = 4).

Discussion

The present results confirm the stimulatory action of prostaglandin E_2 and forskolin on transepithelial sodium transport across renal A6 epithelia. These findings extend the results of previous patch clamp studies of A6 Na^+ channel regulation (Kokko et al., 1994, Marunaka & Eaton, 1991) and previous studies of transepithelial Na^+ transport in native tight epithelia including frog skin (Els & Helman 1981, 1997) and toad urinary bladder (Frazier & Yorio, 1992). A new finding was the strong dependence of prostaglandin and cAMP effects on solution osmolarity. Specifically, PGE_2 and cAMP evoked large increases in I_{sc} and G_t in isosmotic solutions but had little effect in hyposmotic solutions. Therefore, the stimulation of sodium transport by hyposmotic solutions, PGE_2 and cAMP apparently involves interacting mechanisms. These findings contrast with mineralocorticoid stimulation of renal A6 epithelial sodium transport which resulted in a twofold increase in I_{sc} in A6 epithelium in both isosmotic and hyposmotic solutions (Wills et al., 1993 and *unpublished observations*). We note that recent evidence for an independent action of mineralocorticoids and PGE_2 was also reported by Paunescu & Helman 1997). When hyperosmotic solutions were used, no stimulation of transport was evoked by PGE_2 or forskolin (*present results*), or by aldosterone (N.K. Wills, *unpublished observations*).

Role of PGE_2 in Sodium Transport Regulation

In agreement with previous studies of PGE_2 in native amphibian tight epithelia, (*c.f.* Els & Helman, 1997; Nielsen, 1990; Frazier & Yorio, 1992), PGE_2 stimulated sodium transport across cultured renal A6 epithelia as evidenced by the nearly twofold increase in amiloride-sensitive conductance and current. However, these effects were attenuated when serosal hyposmotic solutions were employed. Moreover, pretreatment with PGE_2 prevented subsequent stimulation of I_{sc} and G_t in response to serosal hyposmotic challenge. The inhibitory effects of indomethacin provide further support for a role for PGE_2 in regulating renal basal and hyposmotic-induced sodium transport.

Keeler & Wong (1986) reported that PGE_2 addition to A6 cells produced an increase in the amiloride-insensitive I_{sc} that was due to chloride secretion. We did not determine the ions responsible for the amiloride-insensitive current, but it was similar in magnitude to that observed by Keeler and Wong (1986; 2–5 μA/cm^2). In contrast to their findings, we observed a large increase in the amiloride-sensitive current following PGE_2 addition that was present also when the chloride in the bathing solutions was replaced by gluconate (Mo & Wills, 1995 and *unpublished results*). Another difference between the two studies was that the basal I_{sc} values were lower for Keeler and Wong (1986), approximately 4 μA/cm^2, compared to ~15 μA/cm^2 in the present study. One fac-

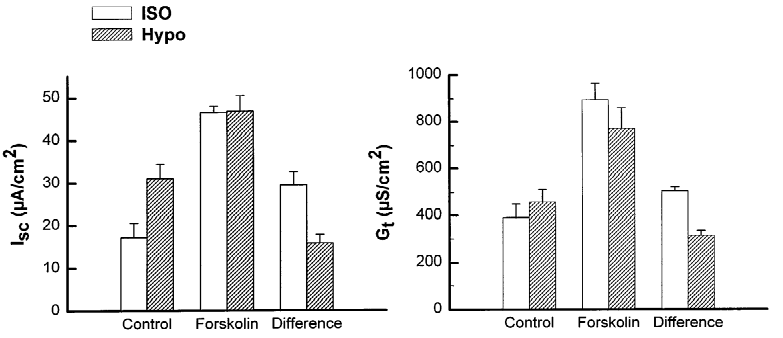

Fig. 9. Comparison of the effects of forskolin (10 μM serosal addition) in isosmotic and hyposmotic serosal bathing solutions ($n = 5$).

Table 2. Effects of IBMX on transepithelial electrical parameters and interactions with hyposmotic solutions

	I_{sc} (μA/cm²)	G_t (μS/cm²)
Control (Isosmotic) $n = 7$	15 ± 1	322 ± 20
IBMX (100 μM) $n = 7$	24 ± 2*	640 ± 45*
" " + Amiloride (50 μM) $n = 4$	1 ± 1*,***	287 ± 18***
Control (Isosmotic)	11 ± 1	268 ± 12
HYPO	39 ± 6*	506 ± 32*
HYPO + IBMX	44 ± 8*	701 ± 30*,f
" " + Amiloride (50 μM)	1 ± 1*,**	245 ± 21**
$N = 3$ (*paired measurements*)		
Control (Isosmotic)	14 ± 0.2	324 ± 29
IBMX (100 μM)	27 ± 1*	705 ± 8*
IBMX + HYPO	22 ± 1***	738 ± 11*
" " + Amiloride (50 μM)	1 ± 1*,**	254 ± 14**
$N = 3$ (*paired measurements*)		

* $P < 0.05$ compared to control
** $P < 0.05$ compared to Hypo and IBMX combined condition
*** $P < 0.05$ compared to IBMX condition
[f] $P < 0.05$ compared to HYPO condition

Table 3. Effects of a PKA inhibitor on the hyposmotic response

	I_{sc} (μA/cm²)	G_t (μS/cm²)
Control		
Isosmotic	22 ± 4	359 ± 46
Hyposmotic[a]	41 ± 2**	525 ± 41**
Difference $N = 4$	20 ± 2	165 ± 26
H89 treatment (10 μM serosal)		
Isosmotic	3 ± 1	241 ± 32
Hyposmotic[a]	6 ± 2	296 ± 43
Difference $N = 4$	3 ± 1*	56 ± 13*

[a] Isosmotic solution mucosal, hyposmotic solution serosal
* $P < 0.05$ compared to control; **$P < 0.05$ compared to isosmotic

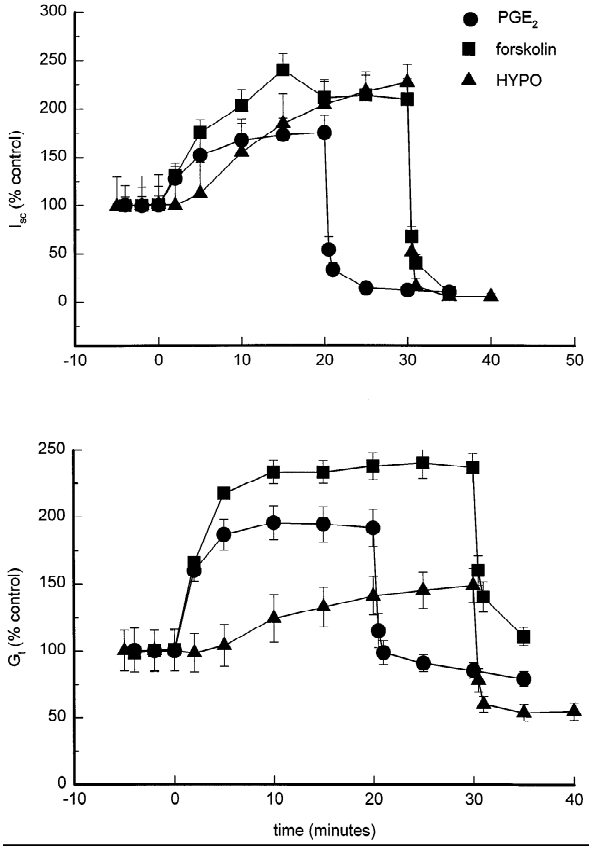

Fig. 10. Comparison of the time course of the effects of PGE_2, forskolin (FSK), and serosal hyposmotic challenge (HYPO). Data are from Figs. 1, 2, and 8 and were normalized to control values at $t = 0$, the time of the onset of the experimental manipulations. Amiloride was added to the mucosal bath at $t = 20$ min for PGE_2, and at $t = 30$ min for HYPO and FSK.

tor that might explain this discrepancy in the basal and PGE_2-induced I_{sc} is the different osmolalities of the solutions used in the studies. In the present study, I_{sc} was decreased in hyperosmotic solutions and the PGE_2-induced stimulation of the amiloride-sensitive current, but not the amiloride-insensitive current, was attenuated in hyperosmotic solutions. The solutions used by Keeler and Wong (1986) had a higher osmolality (240 mosmol/kg H_2O) than our study (200 mosmol/kg H_2O).

PGE_2 normally has an inhibitory effect on amiloride-sensitive Na^+ transport in rabbit cortical collecting

duct unless Na^+/Ca^{2+} exchange across the basolateral membrane is blocked (Stokes & Kokko, 1977; Hébert, Jacobson & Breyer, 1991*a*). When Na^+/Ca^{2+} exchange is abolished, PGE_2 stimulates Na^+ transport (Hébert et al., 1991*b*). Kokko et al. (1994) have suggested that A6 cells might possess a less active basolateral membrane Na^+/Ca^{2+} exchange or that the activity of this exchanger is not modulated by cAMP in A6 cells. In their patch clamp studies of isolated apical membrane sodium channels in A6 cells, a complex pattern of regulation emerged. Channel activity was initially inhibited by PGE_2 for 1–6 min followed by stimulation of channel activity.

In the present study, rapid inhibitory effects of PGE_2 on the short-circuit current or the transepithelial conductance were not observed. One possible reason for this difference is that the rate of basal sodium transport may be an important factor in determining the time course of PGE_2 effects. Punescu and Helman (1997) reported that the onset of PGE_2 stimulation of I_{sc} was more rapid in aldosterone-treated A6 epithelia with high sodium transport rates. Consequently, it is possible that the high basal transport rates of the tissues in the present study may have resulted in a rapid stimulation of I_{sc} that obscured detection of such inhibitory events.

The stimulatory actions of PGE_2 were apparently unique to this particular prostaglandin since other prostaglandins found in the kidney, specifically $PGF_{2\alpha}$ or PGI_2, (Morrison, 1986; Frazier & Yorio, 1992) were ineffective. There are three major groups of PGE_2 receptors, each of which produces a different effect (Hébert et al., 1991*b*). The effects of PGE_2 in our studies are probably due to the receptor that stimulates adenyl cyclase. Four observations support this hypothesis. First, PGE_2 increases cAMP in A6 cells (Kokko et al., 1994). Second, H89 significantly attenuated the actions of PGE_2. In addition, forskolin, an adenyl cyclase activitor, had qualitatively similar effects as PGE_2 on G_t and I_{sc}. Finally, the interactions between hyposmotic solutions and PGE_2 or forskolin, were similar.

Factors Governing the Generation of PGE_2

As discussed previously, PGE_2 is generated by the metabolism of arachidonic acid (AA). A major route for AA synthesis depends on PLA_2 (Feinstein & Sha'afi, 1983; Frazier & Yorio, 1992). In our studies, inhibition of PLA_2 significantly attenuated the hyposmotic stimulation of I_{sc} and G_t. Preliminary results show that inhibition of phospholipase C (PLC) and diacylglycerol lipase, an alternative pathway to produce AA (Feinstein & Sha'afi, 1983; Frazier & Yorio, 1992), did not significantly affect the hyposmotic stimulation of sodium transport (*data not shown*). These results suggest that PLA_2, but not PLC/diacylglycerol lipase, has a role in the hyposmotic stimulation of sodium transport in A6 cells.

Table 4. Effects of hyperosmotic serosal solution on the forskolin response

	I_{sc} ($\mu A/cm^2$)	G_t ($\mu S/cm^2$)
Control (Isosmotic)	23 ± 4	469 ± 42
Hyperosmotic[a]	1 ± 0.3*	309 ± 31*
" " + Amiloride[b] $n = 4$	1 ± 0.1	359 ± 57
Control (Isosmotic)	20 ± 2	425 ± 41
Forskolin[c]	40 ± 4*	784 ± 90*
Hyperosmotic + Forskolin[c]	7 ± 1**	384 ± 34**
" " + Amiloride[b] $n = 4$	2 ± 0.5***	311 ± 28

[a] Hyperosmotic solution contained 120 mM mannitol; Osmolality = 290 mosm/kg H_2O
[b] 50 μM Amiloride added to mucosal solution
[c] 10 μM Forskolin added to serosal solution
* $P < 0.05$ compared to control condition
** $P < 0.05$ compared to forskolin condition
*** $P < 0.05$ compared to hyperosmotic and forskolin combined condition

Adenyl Cyclase, cAMP and the Role of Protein Kinase A

A number of prostaglandins produce their effects by increasing cAMP (Pohlman et al., 1983; Yanase & Handler, 1986*b;* Kokko et al., 1994) which leads to the activation of protein kinase A (PKA) and protein phosphorylation. Consequently it is likely that cAMP/PKA may partially mediate the effects of PGE_2 or hyposmotic solutions. In this regard, it is notable that hypotonic solutions increase cAMP in lymphoma cells (Watson, 1990) and A6 cells (P.S. Matsumoto, L. Mo & N.K. Wills, *unpublished observations*). In the present study, H89, a PKA inhibitor, reduced the stimulation of G_t and I_{sc} by a hyposmotic solution. Furthermore, forskolin, an activator of adenyl cyclase stimulated I_{sc} and G_t and attenuated the effects of a hyposmotic solution on G_t and I_{sc}.

Two aspects of these results suggest a role for cAMP and PKA in regulating basal sodium transport as well. First, addition of IBMX (100 mM serosal bath) produced an increase in I_{sc} and G_t ($\Delta I_{sc} = 11 \pm 2$ $\mu A/cm^2$ and $\Delta G_t = 355 \pm 28$ $\mu S/cm^2$; $n = 4$) that was blocked by amiloride (*see* Table 2). Second, the PKA inhibitor H89 reduced the basal rate of sodium transport for epithelia bathed in symmetric isosmotic solutions. The stimulation of I_{sc} by IBMX may reflect an elevation of intracellular cAMP due to inhibition of phosphodiesterase as reported by Nisato and Marunaka (1997), although Kokko et al. (1994) did not observe an increase in intracellular cAMP levels in IBMX-treated tissues. The reasons for this discrepancy are unknown. Nonetheless, we note that the effects of forskolin, an agent known to increase cAMP, were attenuated in hyperosmotic solutions (Table 4) consistent with an interaction of solution osmolarity and intracellular cAMP levels.

Membrane Localization of the Effects Hyposmotic Solutions, PGE_2 and Forskolin

Increased transepithelial sodium transport could potentially result from changes in basolateral membrane properties, specifically, the basolateral membrane potassium conductance. However, in previous studies (Wills et al., 1991), we found that changes in basolateral membrane properties were too small to account for the approximately twofold increase in sodium transport (measured as I_{sc}) evoked by hyposmotic challenge. In the present study I_{sc} increased by ~100% after forskolin (FKS) addition, ~120% in the presence of PGE_2, and ~140% following exposure to hyposmotic solutions. Since $I_{sc} = G_c \cdot E_c$, a 100% increase in I_{sc} would require either a twofold increase E_c, i.e., from ~100 to ~200 mV or a comparable increase in G_c. For changes in the basolateral membrane driving force to sufficiently increase E_c, intracellular potassium activity levels would have to reach unphysiologically high levels. Similarly, changes in the basolateral membrane resistance (R_b) cannot adequately support a twofold increase in G_c, since R_a (apical membrane resistance) $\gg R_b$ (Wills et al., 1992) and $G_c = 1/(R_a + R_b)$. Consequently, the effects of the above agents are likely to reflect changes in the apical membrane sodium conductance.

Implications for Osmotic Regulation of Sodium Transport

As discussed above, previous studies from this laboratory have shown that sodium channel activity is stimulated by serosal hyposmotic solutions and inhibited by hyperosmotic solutions. The hyposmotic stimulation of sodium transport is not due to a ''direct'' mechanical effect, such as stretch activation of a (nonadapting) mechanosensitive sodium channel since the largest cell volume and maximum value for sodium transport do not occur at the same time (Crowe et al., 1995). More specifically, cell volume reached a maximum value within ~5 min and then decreased over the next 20 min to a value slightly higher (~8%) than the initial control level. In contrast, G_T and I_{sc} showed a gradual increase within 1–2 min of the onset of the hyposmotic challenge, and continued to increase even during the regulatory volume decrease, reaching a new steady-state within 25–30 min.

The time lag between the maximum cell volume change and the maximum increase in I_{sc} evoked by hyposmotic solutions suggests mediation of these effects by a cascade of intracellular signaling events. Forskolin and PGE_2 are both known to activate intracellular signaling pathways, yet the responses to these agents are much faster than the response to a hyposmotic solution (Fig. 10). Indeed, the results of pharmacological inhibitors of the production of cAMP and AA metabolites, and PGE_2, suggest that these agents might, in fact, participate in the hyposmotic response.

In contemplating a possible role for cAMP and PGE_2 in the osmotic regulation of sodium channels, it is tempting to speculate that cell swelling induced by hyposmotic solutions could result in an increase in intracellular calcium, possibly leading to activation PLA_2, which would in turn increase the production of AA. Metabolism of AA partially into PGE_2 could lead to increases in intracellular cAMP. Alternatively, activation of adenyl cyclase activity could also directly increase intracellular cAMP. Cyclic AMP would then activate PKA, resulting in an increase in sodium transport. Consistent with this model, Ehrenfeld and coworkers (Ehrenfeld, Raschi & Brochiero, 1994; Brochiero, Raschi & Ehrenfeld, 1995) have shown that intracellular calcium is increased following hyposmotic shock. In addition, preliminary measurements of cAMP indicate that this parameter is also increased following hyposmotic challenge (P.S. Matsumoto, L. Mo, & N.K. Wills, *unpublished observations*).

Unlike the effects of hyposmotic solutions, PGE_2 or cAMP also increase amiloride-insensitive currents and conductances in A6 cells. Although, we cannot presently explain this difference, it is conceivable that hyposmotic challenge could cause the release or generation of factors that are antagonistic to the effects of PGE_2 or cAMP on the amiloride-insensitive pathway. For example, the production of arachidonic acid (AA) by PLA_2 has been shown to inhibit epithelial chloride conductances (Mochizuki, Chao & Widdicombe, 1992). Consequently, simultaneous generation of AA and PGE_2 or cAMP might have no net effect on the amiloride-insensitive pathway while stimulating transepithelial sodium transport.

Regardless of the precise role of prostaglandins and cAMP in sodium channel regulation, the above findings suggest a hierarchical role for osmotic activation (or deactivation) of sodium channel activity. Hyposmotic solutions, prostaglandins and cAMP all lead to elevations in sodium channel activity, perhaps by a similar mechanism of channel recruitment, e.g., by conformational changes or insertion of additional channels into the membrane. Once fully ''recruited'' or available, the channels are apparently able to undergo further activation by other regulators such as aldosterone. Conversely, in the presence of hyperosmotic solutions, the channels or perhaps the population of conducting channels become unavailable and efforts to stimulate the channels are ineffective.

Clearly, further experiments including knowledge of channel structure and rates of channel turnover in the apical membrane are needed to test the above hypotheses. Nonetheless, the present results indicate the importance of solution osmolality as a potential variable in studies of hormones and intracellular messenger regula-

tion of sodium channel activity. Previous studies have demonstrated interactions between cell swelling and cAMP in the regulation of chloride channels in a number of cell types, including hepatocytes (Meng & Weinmann, 1996) and atrial cells (Sorato, 1992). Therefore, such interactions may be a general feature of some types of cAMP regulated channels.

In conclusion, the present results indicate that plasma osmolality can be a potent variable in determining the effects of other sodium transport regulators. Regional variations in plasma or interstitial osmolality normally occur in the lung and kidney, and alterations in osmolality can also arise in pathological conditions such as water intoxication, hemorrhagic shock, or pulmonary edema. Consequently, it may be important clinically to understand the interactive roles of these signaling pathways as determinants of sodium and volume homeostasis.

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