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Abstract

The effect of adenosine on Na+/H+ exchange activity was examined in cultured A6 renal epithelial cells. Adenosine and its analogue N6-cyclopentyladenosine (CPA) had different effects on Na+/H+ exchange activity depending on the side of addition. Basolateral CPA induced a stimulation of Na+/H+ exchange activity that was completely prevented by preincubation with an A2A-selective antagonist, 8-(3-chlorostyryl)caffeine, whereas apical CPA induced a slight but significant inhibition of Na+/H+ exchange activity that was significantly reduced by the A1-receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine. Protein kinase C activation may be involved in mediating the apical CPA inhibition of Na+/H+ exchange activity; this inhibition was prevented by the protein kinase C inhibitor calphostin C. Treatment with either forskolin or 8-bromo-cAMP significantly stimulated Na+/H+ exchange activity; only basolateral CPA addition induced an increase in cAMP level. These observations together with the finding that the CPA-dependent stimulation of exchange activity was prevented by the protein kinase A inhibitor H-89 support the hypothesis that basolateral CPA stimulates Na+/H+ exchange via adenylate cyclase/protein kinase A activation. Basolateral CPA also increased transepithelial Na+ transport, and this stimulation was prevented by the Na+/H+ exchange inhibitor HOE-694, suggesting that changes in pHi during hormone action can act as an intermediate in the second-messenger cascade.

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Polarization of Adenosine Effects on Intracellular pH in A_6 Renal Epithelial Cells

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SUMMARY

The effect of adenosine on Na^+/H^+ exchange activity was examined in cultured A_6 renal epithelial cells. Adenosine and its analogue N^6-cyclopentyladenosine (CPA) had different effects on Na^+/H^+ exchange activity depending on the side of addition. Basolateral CPA induced a stimulation of Na^+/H^+ exchange activity that was completely prevented by preincubation with an A_{2A}-selective antagonist, 8-(3-chlorostyryl)caffeine, whereas apical CPA induced a slight but significant inhibition of Na^+/H^+ exchange activity that was significantly reduced by the A_1-receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine. Protein kinase C activation may be involved in mediating the apical CPA inhibition of Na^+/H^+ exchange activity; this inhibition was prevented by the protein kinase C inhibitor calphostin C. Treatment with either forskolin or 8-bromo-cAMP significantly stimulated Na^+/H^+ exchange activity; only basolateral CPA addition induced an increase in cAMP level. These observations together with the finding that the CPA-dependent stimulation of exchange activity was prevented by the protein kinase A inhibitor H-89 support the hypothesis that basolateral CPA stimulates Na^+/H^+ exchange via adenylate cyclase/protein kinase A activation. Basolateral CPA also increased transepithelial Na^+ transport, and this stimulation was prevented by the Na^+/H^+ exchange inhibitor HOE-694, suggesting that changes in pH_i during hormone action can act as an intermediate in the second-messenger cascade.

Adenosine, acting as a local hormone, regulates a number of renal functions, including renal hemodynamics, renin release, and ion transport, via several types of cell surface receptors that have been differentiated on the basis of their affinity for various adenosine analogues and their effect on adenylate cyclase (1, 2). The A_1 receptor has a high affinity for adenosine and inhibits adenylate cyclase while stimulating phosphoinositide turnover and mobilizing intracellular calcium; the A_2 receptor has a lower affinity for adenosine and stimulates adenylate cyclase (3). A_2 receptors are further divided into A_{2A} (high affinity for agonists) and A_{2B} (lower affinity) subtypes (4). Both A_1 and A_2 receptors are widely distributed throughout the nephrons as well as in several renal cell lines derived from different nephron segments (5–10). The ability of adenosine to couple to different effector systems is believed to account for its pleiotropic actions in renal cells. We recently demonstrated that A_6 cells, a cell line derived from the kidney of *Xenopus laevis* (11) that is commonly used as a model of the mammalian collecting duct, contain both A_1 and A_2 receptors (12). The A_1 receptors are located on the apical surface and regulate apical Cl^- secretion via intracellular calcium, whereas A_2 receptors are located on the basolateral surface and stimulate Na^+ transport via an increase in cAMP intracellular levels. The expression of both adenosine receptors capable of regulating different ion transports on the same cell suggests a dual-control nature of adenosine as a regulator of kidney cell function.

Alterations in pH_i have been demonstrated to modulate the action of different hormones on apical Na^+ conductive transport in tight epithelia (13–16). Na^+/H^+ exchange is a major determinant of pH_i and may be regulated by various hormones; it is mainly regulated by the phospholipase C/PKC and adenylate cyclase/PKA signaling pathways (17). We previously demonstrated that A_6-2F3 cell monolayers contain a basolaterally located Na^+/H^+ exchanger that is presumed to be involved primarily with cellular pH regulation (18). The current study was performed to determine whether changes in pH_i via regulation Na^+/H^+ exchange activity act as an intermediate in the second-messenger cascade initiated by

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ABBREVIATIONS: pH_i, intracellular pH; CPA, N^6-cyclopentyladenosine; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; DPMA, N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine; HOE-694, (3-methylsulfonyl-4-piperidino-benzoyl)guanidine methanesulfonate; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]5-isoquinolinesulfonamide; TPA, phorbol-12-myristate-13-acetate; TMA, tetramethylammonium; CSC, 8-(3-chlorostyryl)caffeine; CPX, 1,3-dipropyl-8-cyclopentylxanthine; PKC, protein kinase C; PKA, protein kinase A; I_{sc}, short circuit current; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

adenosine and ending in a modification of apical Na^+ conductance. The availability of potent selective adenosine A_1 and A_2 receptor antagonists and agonists provides the pharmacological probes by which the role of A_1 and A_2 receptors can be investigated in polarized A_6 cell monolayers. Our results indicate that adenosine most likely stimulates Na^+/H^+ exchange activity by increasing intracellular cAMP via activation of basolateral A_2 receptors. This intracellular alkalinization may play a permissive role in inducing the observed increase in transepithelial Na^+ transport induced by basolateral adenosine.

Experimental Procedures

Cell culture. Experiments were performed with A_6 cells from the A_6-C1 subclone (passage 114–128). This subclone was obtained by ring-cloning of A_6-2F3 cells at passage 99 and was selected for its high transepithelial resistance and responsiveness to aldosterone and antidiuretic hormone (19).

Cells were cultured in plastic culture flasks at 28° in 5% CO_2 atmosphere in 0.8× concentrated Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 25 mM $NaHCO_3$ and supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Irving, UK) and 1% of a penicillin/streptomycin mix (Seromed, Berlin, Germany) (final osmolality, 230–250 mOsmol). No supplemental aldosterone was added to the medium. Cells were subcultured weekly via trypsinization into a Ca^{2+}/Mg^{2+}-free salt solution containing 0.25% (w/v) trypsin and 1 mM EGTA and then diluted into the above growth medium.

For all experiments, cells were plated onto permeant filter supports (O.4-μm pore size, 4.7 cm^2, Transwell; Costar, Cambridge, MA) previously coated with a thin layer of rat tail collagen (Biospa) according to the method of Krayer-Pawlowska *et al*. (19). Experiments were usually performed 10–15 days after seeding, and the monolayers were fed three times a week. Fresh medium was always given the day before the start of the experiment.

Fluorescence measurements. pH_i was measured using the pH-sensitive fluorescent dye BCECF. Cells on permeable supports were loaded with the acetoxymethyl ester derivative of BCECF (10 μM) for 60 min at room temperature in sodium medium. To avoid dye leakage, BCECF loading was carried out in the presence of 50 μM probenecid. Coverslips with confluent monolayers were inserted at an angle of 60° in a fluorometer cuvette designed for independent perfusion of the apical and basolateral cell surfaces as previously described (20).

Fluorescence was recorded with a Shimadzu RF 5000 spectrofluorometer using 535 nm (bandwidth, 20 nm) as emission wavelength and 500 nm (pH sensitive) and 440 nm (pH insensitive) as excitation wavelengths (bandwidth for each, 5 nm). pH_i was calculated from the ratios with fluorescence intensities at the two above-mentioned excitation wavelengths by using a standard calibration procedure based on the use of nigericin in high K^+ media buffered at different pH values, as previously described (20).

The Na^+/H^+ exchange activity was investigated by monitoring pH_i recovery after an acid load by using the NH_4Cl prepulse technique (21). The rate of Na^+-dependent alkalinization was determined by linear regression analysis of 15 points taken at 4-sec intervals. A similar number of data points were collected in all recoveries examined. The use of nominally CO_2/HCO_3^--free solutions minimizes the likelihood that Na^+-dependent HCO_3^- transport was responsible for the observed pH_i changes. The Na^+-dependent alkalinization in each experiment was always examined from the same starting pH_i value because Na^+/H^+ exchange activity is under the influence of pH_i.

Adenosine agonists and antagonists. To distinguish between the involvement of the putative adenosine receptor subtypes in adenosine regulation of Na^+/H^+ exchange activity and cAMP generation in A_6 cells, we used various adenosine agonists and antagonists with the following K_i values reported in different mammalian tissues (for a review, see Ref. 4): CPA, A_1 = 0.6 nM versus A_{2A} = 460 nM; DPMA, A_1 = 140 nM versus A_{2A} = 4.4 nM, CPX, A_1 = 0.9 nM versus A_{2A} = 470 nM versus A_{2B} = 360 nM; and CSC, A_1 = 28,000 nM versus A_{2A} = 54 nM versus A_{2B} = 8,200 nM.

Composition of perfusion fluids. All pH_i measurements were performed at room temperature in HEPES-buffered media. Sodium medium contained 110 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 mM KH_2PO_4, 5 mM glucose, and 10 mM HEPES buffered with Tris to final pH 7.5. For cellular acidification, we used an NH_4Cl solution identical to Na^+ medium plus 20 mM NH_4Cl. A sodium-free (TMA medium) was obtained by complete replacement of sodium with TMA.

cAMP determination. Intracellular cAMP levels were analyzed as previously reported (12, 22, 23). Cell monolayers grown on filter inserts were placed in the A_6 Ringer's solution described above and exposed to hormones for 15 min in the presence of 1 mM rolipram, a phosphodiesterase inhibitor that is not an adenosine receptor antagonist. When used, the adenosine antagonists were added 5 min before the addition of adenosine. The monolayers were rapidly rinsed twice with ice-cold assay buffer (50 mM Tris·HCl, 16 mM 2-mercaptoethanol, 8 mM theophylline, pH 7.4) and immediately immersed in liquid nitrogen. The filter apparatus was stored at −20° until assayed. For assay, the filters were cut out of the filter apparatus while still frozen and immersed in 100 μl of the above assay buffer plus 10 μl of 0.1 M HCl in an Eppendorff tube. Cells were disrupted by two 5-sec pulses with a probe sonicator (Branson, Zurich, Switzerland), the sample was neutralized with 10 μl of 0.1 N NaOH, and the filter and cell debris were removed by centrifugation at 14,000 rpm for 15 sec in an Eppendorff centrifuge. The cAMP concentration was determined of a 50-μl aliquot of the supernatant using the test kit from DuPont-New England Nuclear (Boston, MA) based on a competitive protein-binding assay (24).

Measurements of transepithelial I_{sc}. Measurements of transepithelial potential difference (mV) and I_{sc} ($\mu A/cm^2$) were performed in a modified chamber according to published methods (12). Transepithelial resistance (½ × cm^2) was calculated according to Ohm's law; the electrical parameters were measured in Na^+ medium used for pH_i measurements at room temperature.

Materials. BCECF-AM was purchased from Molecular Probes (Eugene, OR). HOE-694 was kindly provided by Dr. H. J. Lang (Hoerchst AG, Frankfurt, Germany). H-89 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA) and calphostin C from Calbiochem (San Diego, CA)and activated according to the manufacturer's instructions. All other substances were obtained from Sigma Chemical (St. Louis, MO).

Data analysis and statistics. Data are expressed as mean ± standard error. Statistical comparisons were made using the paired and unpaired Student's t tests, and $p < 0.05$ indicated a statistical difference.

Results

Electrical parameters. The effect of adenosine was studied in confluent A_6 monolayers grown onto porous filters, which allows selective access to the basolateral and apical sides of the epithelial cell. After 10–14 days of growth, A_6 monolayers displayed a high transepithelial resistance (9850 ± 467 ½ × cm^2, 40 experiments) and substantial I_{sc} (5–10 $\mu A/cm^2$). These parameters were always controlled before Na^+/H^+ exchange activity was measured to ensure that the monolayers examined were "tight."

Effect of CPA on Na^+/H^+ exchange activity. In a previous study, we found that A_6 cells recover from an acid load by activation of a Na^+/H^+ exchanger located exclusively on the basolateral side that was highly sensitive to the addition

of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (18). To study the effect of adenosine on Na^+/H^+ exchange activity of the A_6 cell monolayers, we used CPA, a metabolically stable adenosine analogue poorly taken up by cells that binds both A_1 and A_{2A} adenosine receptor subtypes but has a higher affinity for the A_1 receptor (25).

We first analyzed the cell surface polarity of the effect of 10^{-6} M CPA on Na^+/H^+ exchange activity. Fig. 1 shows a typical experiment in which the A_6 monolayers were acidified by NH_4Cl prepulse and recovery was monitored in the presence of basolateral Na^+ buffer before and after the addition of CPA to the apical (Fig. 1A) or basolateral (Fig. 1B) side of A_6 monolayers. We analyzed pH_i recovery at a submaximal Na^+ concentration (22 mM) to better observe the variation in rate of recovery. As indicated in the figure, after replacement of NH_4Cl medium by TMA (data collection started immediately after this solution change), cellular pH_i dropped. A rapid recovery of pH_i ensued only when Na^+-free buffer was replaced by Na^+ buffer in the basolateral fluid compartment. A preincubation period of 20 min with apical CPA slightly inhibited the pH_i recovery (0.511 ± 0.087 versus 0.425 ± 0.090 ΔpH/min, $p < 0.001$, five experiments), whereas basolateral incubation induced a strong increase in the Na^+/H^+ exchange activity (0.513 ± 0.067 versus 0.715 ± 0.100 ΔpH/min, respectively, $p < 0.001$, five experiments). Because the Na^+-dependent pH_i recovery was always initiated at a similar pH_i, a change in ΔpH/min after the CPA addition is expected to most likely be a consequence of CPA-induced alterations of the transport process itself and not a consequence of different cellular acid loads (allosteric control of Na^+/H^+ exchange). Data from all experiments that were performed under these conditions are summarized in Fig. 2, in which it can be seen that 10^{-6} M CPA significantly stimulated the Na^+/H^+ exchange activity when it was added to the fluid perfusing the basolateral side of the A_6 monolayer, whereas apical CPA slightly, but significantly, inhibited the Na^+/H^+ exchange activity. Similar results were obtained using natural adenosine (data not shown); however, CPA, although less potent than adenosine, gave more homogeneous results, probably because of its lower rate of metabolic breakdown.

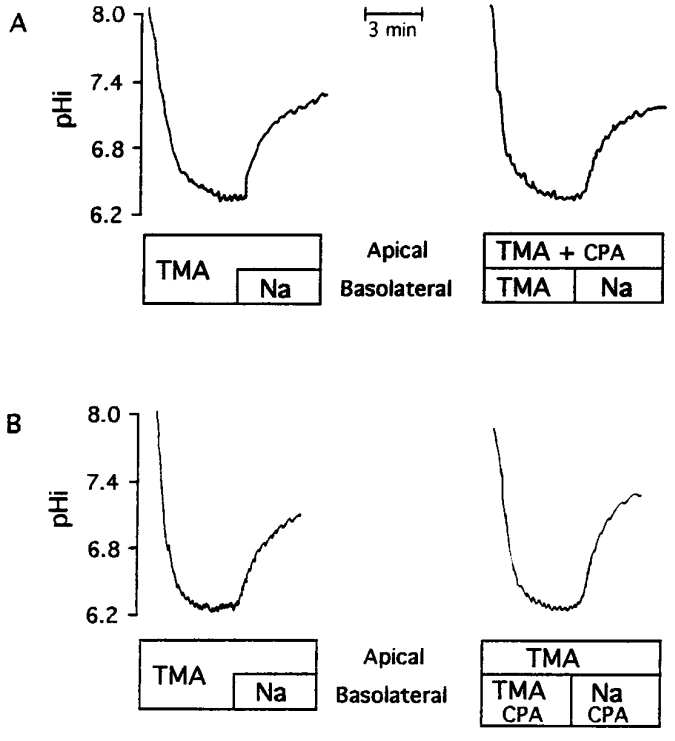

Fig. 1. Representative traces depicting the effect of 10^{-6} M CPA on Na^+/H^+ exchange activity in A_6 monolayers. Na^+/H^+ exchange activity was assayed as the initial rate of Na^+-dependent pH_i recovery induced by the basolateral addition of 22 mM Na^+ after intracellular acidification via superfusion with NH_4^+ medium followed by Na^+-free TMA medium (see Experimental Procedures). First trace, absence of CPA. Second trace, obtained in the same monolayer after a 20-min preincubation with CPA. A, CPA was added only to the apical side. B, CPA application was only to the basolateral side of the monolayer. *pH_i* corresponds to the nigericin calibration performed as described in Experimental Procedures.

Because the extracellular free adenosine concentration found in the kidney ranges from 10^{-8} to 10^{-6} M (5), we analyzed the concentration dependence of basolateral CPA action on Na^+/H^+ exchange activity from 10^{-9} to 10^{-6} M. As shown in Fig. 3, a 15-min basolateral CPA preincubation caused a dose-dependent increase in the Na^+/H^+ exchange activity.

We then analyzed the basolateral CPA-dependent stimulation with increasing extracellular Na^+ concentrations (Fig. 4). Assuming a simple Michaelis-Menten relationship and using a nonlinear least-squares fit program, we derived the K_m value for Na^+ in the absence and presence of basolateral 10^{-6} M CPA by following the protocol used for Fig. 1. Basolateral CPA treatment reduced the apparent K_m value for external Na^+ from 40 ± 5.6 to 12 ± 3.9 mM. Consequently, it seems that at least part of the observed effect of CPA when present in the basolateral perfusion fluid can be explained by a change in the Na^+ affinity of the exchanger.

Effects of A_1 and A_2 antagonists on CPA action. To determine whether the stimulatory effect of basolateral CPA occurred via a receptor-mediated mechanism, we examined the effect on CPA action of either the A_1-selective receptor antagonist CPX (4, 25) or the A_{2A}-selective antagonist CSC (4, 26). Neither 10^{-7} M CPX nor 10^{-7} M CSC added alone to the basolateral side of the monolayers altered the Na^+/H^+ exchange rate (data not shown). The basolateral CPA-dependent stimulation of exchange activity was completely pre-

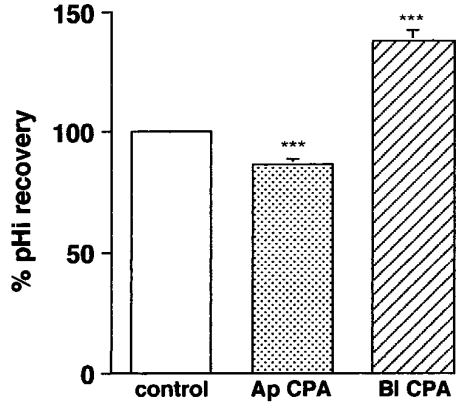

Fig. 2. Effect of apical or basolateral CPA addition on the Na^+/H^+ exchange activity. Experiments were performed according to the protocol described in the legend to Fig. 1. Data are presented as percentage of control values. *Columns*, mean ± standard error of five experiments. ***, $p < 0.001$.

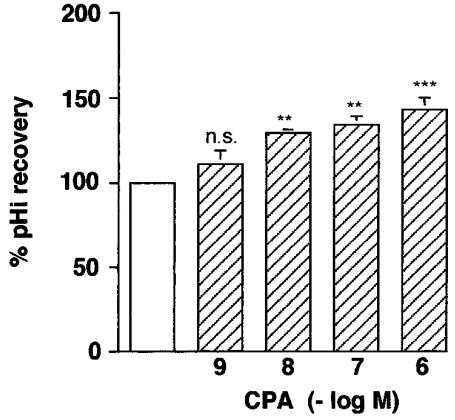

Fig. 3. Effect of increasing concentration of CPA on Na^+/H^+ exchange activity. Experiments were performed at increasing concentrations of CPA according to the protocol described in the legend to Fig. 1. The basolateral side of A_6 monolayers was exposed to CPA for 20 min before analysis of pH_i recovery from an acid load. *Columns*, mean ± standard error of the experiments made at different CPA concentrations (10^{-6} M, seven experiments; 10^{-7} M, three experiments; 10^{-8} M, three experiments; 10^{-9} M, four experiments). **, $p < 0.01$; ***, $p < 0.001$.

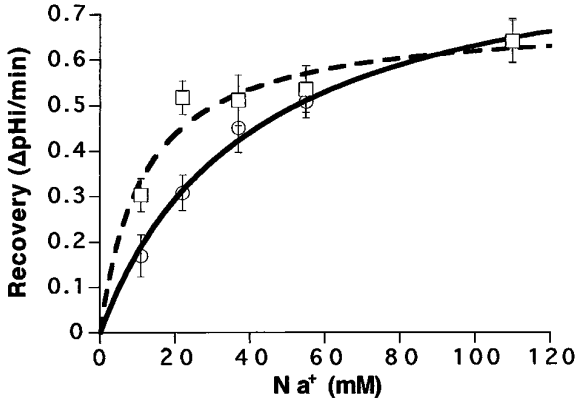

Fig. 4. Effect of various Na concentrations on CPA-dependent stimulation of Na^+/H^+ exchange. Experiments were performed according to the protocol described in the legend to Fig. 1 at different sodium concentrations (11 mM, seven experiments; 22 mM, five experiments; 37 mM, three experiments; 55 mM, seven experiments; 110 mM, six experiments) in the absence (□) or presence (○) of 10^{-6} M CPA. Na^+ activation of basolateral Na^+/H^+ exchange followed simple Michaelis-Menten kinetics. The apparent K_m values were 40 ± 5.6 mM Na^+ in the absence and 12 ± 3.9 mM Na^+ in the presence of CPA .

vented by CSC preincubation, whereas it was not affected by CPX preincubation (Fig. 5). These data suggest that the stimulatory effect of basolateral CPA on Na^+/H^+ exchanger occurs through an A_{2A}-like adenosine receptor localized on the basolateral membrane. Further support for this hypothesis comes from the observation that the A_{2A}-selective agonist DPMA (27), when added to the basal side solution, stimulated Na^+/H^+ exchange activity (0.650 ± 0.069 versus 0.818 ± 0.083 ΔpH/min before and after 10^{-6} M DPMA addition, respectively, $p < 0.05$, four experiments), whereas it had no effect when added to the apical side (data not shown).

We have previously shown in A_6 monolayers that A_2 adenosine receptors are mainly located on the basolateral surface, whereas the A_1 receptors are mainly located on the apical surface (12). Therefore, to verify that the inhibition induced by apical CPA was mediated by A_1 receptors, 10^{-7} M CPX

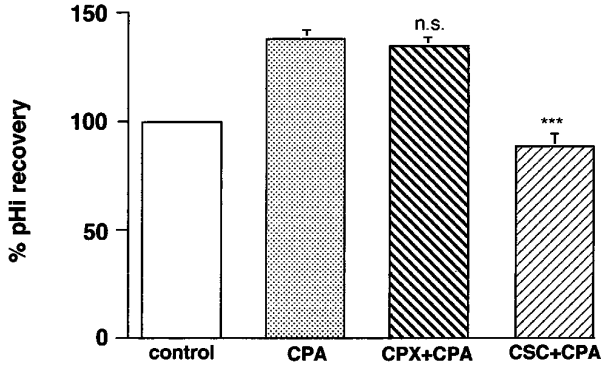

Fig. 5. Effect of the A_1-selective antagonist CPX and the A_{2A}-selective antagonist CSC on basolateral CPA stimulation of the Na^+/H^+ exchanger. Experiments were performed according to the protocol described in the legend to Fig. 1. The A_1 adenosine-selective antagonist CPX (10^{-7} M) or the A_{2A} antagonist CSC (10^{-7} M) was added to the basal solution 5 min before stimulation with CPA (10^{-6} M). *Columns*, mean ± standard error of five experiments. ***, $p < 0.001$, compared with the value for CPA alone.

was added to the apical side of the monolayer 10 min before CPA application. Under these conditions, the inhibition induced by apical CPA was partially but significantly reduced [−16.80 ± 1.73% (five experiments) in the absence versus −5.06 ± 0.82% (four experiments) in the presence of CPX, $p < 0.01$). These experiments confirm the topographic separation of the A_1 and A_2 adenosine receptors that has been previously reported (12).

Second messengers involved in CPA action. To elucidate the signal transduction pathway by which CPA affects pH_i, we analyzed the effect of the pharmacological activation of several regulatory pathways on Na^+/H^+ exchange activity. As shown in Fig. 6, a 5-min preincubation with TPA (10^{-7} M), a phorbol ester known to activate PKC, induced a small but significant inhibition of Na^+/H^+ exchange rate (from 0.506 ± 0.043 to 0.366 ± 0.031 ΔpH/min before and after TPA treat-

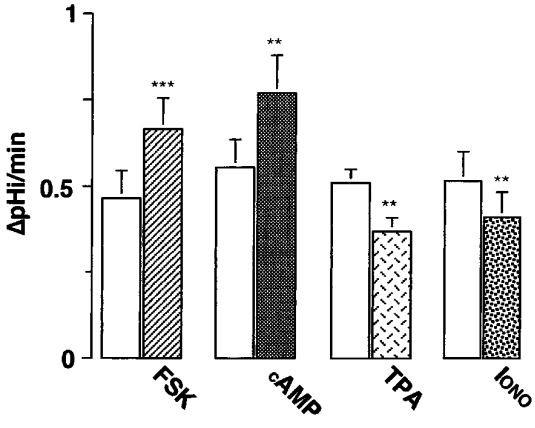

Fig. 6. Effect of the the pharmacological activation of several regulatory pathways on Na^+/H^+ exchange activity. Paired measurements of initial rate of pH_i increase induced by the basolateral superfusion of 22 mM Na^+ medium to acid-loaded cells in the absence (control; *open columns*) or presence of the indicated agents were performed as described in the legend to Fig. 1. Cell monolayers were exposed to various agents from both the apical and basolateral side for different times: forskolin (*FSK*) and 8-bromo-cAMP for 10 min, ionomycin (*Iono*) for 2 min, and TPA for 5 min. Values are mean ± standard error of five experiments for the forskolin, four experiments for 8-bromo-cAMP, three experiments for TPA, and four experiments for ionomycin determinations. **, $p < 0.01$; ***, $p < 0.001$.

ment, respectively, $p < 0.01$, three experiments). Similarly, preincubation with 10^{-6} M ionomycin caused a small inhibition (0.609 ± 0.117 versus 0.493 ± 0.098 ΔpH/min before and after ionomycin, respectively, $p < 0.01$, four experiments), suggesting that Na^+/H^+ exchange in A_6 cells is negatively regulated by both calcium-dependent PKC and intracellular Ca^{2+}. To verify whether PKC is involved in the inhibitory effect of apical CPA on Na^+/H^+ exchange, we analyzed the effect of CPA after pretreatment with calphostin C, a known inhibitor of PKC activity (28). A 5-min preincubation with calphostin C (10^{-8} M) completely prevented the inhibitory action of apical CPA (0.768 ± 0.071 versus 0.774 ± 0.075 ΔpH/min in the absence and presence of CPA plus calphostin C, respectively, p = NS, three experiments) while having no effect on basal Na^+/H^+ exchange rate when added alone to both sides of the monolayers (data not shown). These data support the hypothesis that apical CPA action may be dependent on a functional PKC.

To assess the effect of PKA activation on Na^+/H^+ exchange, first the rate of pH_i recovery was compared in the absence and presence of a cell-permeable analogue of cAMP, 8-bromo-cAMP (5×10^{-4} M), or an agent that stimulates the production of cAMP, forskolin (10^{-5} M). As shown in Fig. 6, both compounds significantly stimulated Na^+/H^+ exchange activity. The amount of the stimulation induced by forskolin or 8-bromo-cAMP exposure (40.48 ± 5.31% and 46.58 ± 6.54%, respectively) was similar to that induced by basolateral CPA treatment (38.20 ± 4.48%), suggesting that basolateral CPA binding to an A_2-like receptor stimulates Na^+/H^+ exchange via adenylate cyclase/PKA. To verify the involvement of PKA in the stimulatory effect of basolateral CPA on Na^+/H^+ exchange, we then analyzed the effect of CPA after pretreatment with H-89, a known inhibitor of PKA activity (29, 30). A 20-min preincubation with H-89 (10^{-6} M) completely prevented the stimulatory action of basolateral CPA (0.523 ± 0.082 versus 0.476 ± 0.055 ΔpH/min in the absence and presence of CPA plus H-89 respectively, p = NS, three experiments) while having no effect on basal Na^+/H^+ exchange rate when added alone to both sides of the monolayers (data not shown). These data support the hypothesis that the stimulatory basolateral CPA action is mediated through the activation of adenylate cyclase/PKA.

To provide additional support for this hypothesis, we determined the effect of basolateral CPA treatment on the levels of intracellular messenger cAMP in cell monolayers in the absence or presence of adenosine antagonists (Fig. 7). Recently, we demonstrated that only basolateral adenosine produced a dose-dependent accumulation of cAMP (12). In Fig. 6, we show that pretreatment with the A_{2A}-selective antagonist CSC markedly inhibited the cAMP increase induced by basolateral CPA (10^{-6} M), whereas the A_1-selective antagonist CPX did not alter this increase. These results together with those obtained from the analysis of the effect of A_1- and A_{2A}-selective adenosine antagonists on basolateral CPA stimulation of Na^+/H^+ exchanger (Fig. 5) suggest that CPA stimulates the Na^+/H^+ exchanger acting through the system adenylate cyclase/PKA by interacting with an adenosine receptor related to the mammalian A_{2A} receptor localized on the basolateral membrane.

Coupling between the pH_i and the transepithelial Na^+ transport. Alterations in pH_i has been reported to directly influence apical Na^+ conductance and/or indirectly

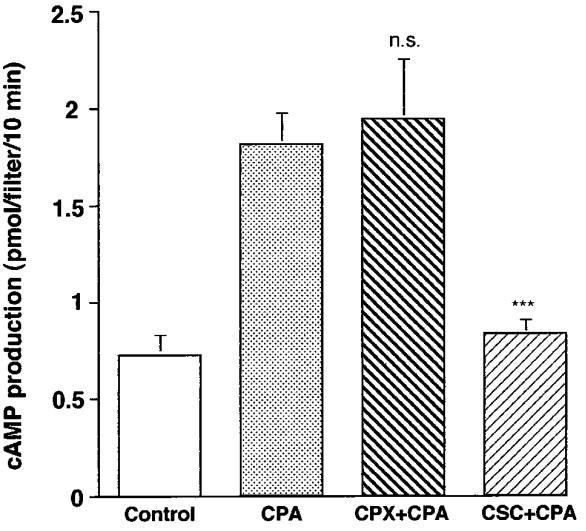

Fig. 7. Effect of preincubation with the antagonists CPX and CSC on the basolateral CPA-induced cAMP generation. A_6 monolayers were preincubated with antagonists 5 min before the addition of CPA (10^{-6} M) to the basolateral side of the monolayers. Levels of intracellular cAMP was determined as described in Experimental Procedures. *Columns*, mean ± standard error of three experiments. ***, $p < 0.001$, compared with the value for CPA alone.

Na^+/K^+-ATPase (13). Our previous observations that basolateral CPA increases Na^+ transepithelial transport in A_6 cell monolayers (12) together with the current findings suggest that the intracellular signal by which adenosine stimulates transepithelial Na^+ transport could be cellular alkalinization via a cAMP-dependent stimulation of Na^+/H^+ exchange. To assess the role of pH_i changes in mediating the action of basolateral CPA on Na^+ transport, we first analyzed the effect of the specific Na^+/H^+ exchanger inhibitor HOE-694 (31) on resting pH_i. We found that although basolateral CPA (10^{-6} M) slightly increased the resting pH_i by 0.16 ± 0.02 units (three experiments), as might be expected from the demonstrated stimulatory action on the Na^+/H^+ exchanger, HOE-694 (2×10^{-7} M) added to the same side of the monolayer induced an intracellular acidification of 0.23 ± 0.02 units (three experiments). We then analyzed the effect of basolateral CPA addition either alone or after HOE-694 preincubation on the transepithelial Na^+ transport measured as I_{sc} in HEPES-buffered solutions (Table 1). Basolateral CPA, as previously observed (12), produced a late and sustained increase in I_{sc} that reached a maximum effect 20 min after the addition of the hormone. The addition of 2×10^{-7} M HOE-694 to the the basal surface of the A_6 monolayers 5 min before the subsequent addition of CPA did not alter basal I_{sc}

TABLE 1

Basolateral CPA effect on short-circuit current in A_6 monolayers

Values are mean ± standard error. CPA (10^{-6} M) was added to the basolateral side of the monolayer. The value reported corresponds to the maximal effect recorder after 20 min of treatment with CPA. HOE-694 (2×10^{-7} M) was added 5 min before CPA treatment. I_{sc}, short-circuit current.

Experimental condition	I_{sc} after CPA/I_{sc} baseline	
	Maximum effect (20 min after CPA)	*n*
CPA	1.96 ± 0.19	6
CPA after HOE-694 preincubation	1.18 ± 0.07[a]	6

[a] $p < 0.001$, paired *t* test.

(9.63 ± 2.51 versus 9.78 ± 2.43 $\mu A/cm^2$ before and after HOE-694 addition, respectively, p = NS, six experiments) while significantly inhibiting the CPA-induced Isc increase. The action of HOE-694 in preventing the CPA-induced I_{sc} increase seems to be due to its specific inhibitory action on the Na^+/H^+ exchanger and not to an interference with A_2 adenosine receptors; CPA-induced intracellular levels of cAMP were not significantly affected by HOE-694 preincubation (1.77 ± 0.2 versus 1.70 ± 0.3 pmol/filter/10 min after CPA plus HOE-694 pretreatment, three experiments).

Discussion

In the current study, we characterized the effect of adenosine on Na^+/H^+ exchange activity in A_6 cell monolayers. We used A_6 cell monolayers as a model to study the mechanism of adenosine action for two reasons. First, the vectorial sodium ion transport in A_6 cells, as in native epithelia such as the mammalian collecting duct or the amphibian skin, is mediated by amiloride-sensitive Na^+ channels at the apical surface and Na^+/K^+-ATPase at the basolateral cell surface (19, 32–34). Second, due to their high tightness (transepithelial resistance, 9850 ± 467 ½ × cm^2), A_6 cell monolayers provide a useful model for the study of the functional localization of adenosine receptor action to the two cell surfaces. In addition, the location and characteristics of Na^+/H^+ exchange activity in A_6 cells have been previously described (18, 22); the Na^+/H^+ exchanger, which is highly sensitive to amiloride inhibition and stimulated by vasopressin, is confined to the basolateral cell surface of A_6 monolayers as observed in cells of rabbit cortical collecting tubule (16, 33, 34).

We assessed adenosine regulation of pH_i by spatially stimulating the A_6 cell monolayers with CPA, a metabolically stable adenosine analogue with higher affinity for the A_1 receptor, used in conjunction with A_1 and A_{2A} receptor-selective antagonists for which the K_i value has been determined in mammalian tissues (see Experimental Procedures). Pharmacological identification of adenosine receptor subtypes has not been fully explored in amphibians (6, 12, 35); therefore, there is the possibility that the selectivity of these agonists and antagonists in A_6 cells could be different than that in mammalian tissues.

The results of the current study demonstrate that basolaterally added CPA caused a consistent stimulation of Na^+/H^+ exchange activity with a consequent significant alkalinization of pH_i. We also observed a small but significant inhibitory effect of CPA on Na^+/H^+ exchange activity when added to the apical side (Figs. 1 and 2). The fact that CPA exerts different effects depending on the side of the addition confirms and extends previous results that demonstrated that A_6 cell monolayers express A_1- and A_2-like adenosine receptors on different cell surface membranes of the same cell that regulate different cell processes: the A_1-like receptors located on the apical membrane regulate chloride secretion via a rapid increase in intracellular calcium, whereas the A_2-like receptors located on the basolateral membrane regulate transepithelial sodium transport via an increase in cAMP (12).

Basolateral CPA action on Na^+/H^+ exchange. We found that the stimulatory effect of basolateral CPA on Na^+/H^+ exchange was dose dependent, with its major effect occurring at a relatively high concentration (10^{-6} M). At this concentration, CPA increased the Na^+/H^+ exchanger's affinity for external sodium without affecting V_{max}. Consequently, it seems that at least a part of the observed basolateral effect of CPA on the pH_i recovery rate can be accounted for by an increased affinity for external sodium. A similar mechanism underlying the vasopressin-dependent increase in Na^+/H^+ exchange activity in A_6 monolayers has also been reported (18).

The fact that the stimulatory effect of basolateral CPA on Na^+/H^+ exchange activity is mediated by the action of basolaterally located adenosine receptors related to the mammalian A_{2A} receptor is supported by the following observations: 1) basolateral CPA-dependent stimulation of the exchanger was completely prevented by the A_{2A} receptor selective antagonist CSC (4, 26), whereas the A_1-selective antagonist CPX (4, 25) had no effect; 2) the A_{2A}-selective agonist DPMA (27), when added to the basal side solution, stimulated Na^+/H^+ exchange activity, whereas it had no effect when added to the apical side; 3) only basolateral CPA stimulated cAMP production, which is known to be associated with A_2 receptor action, and this basolateral CPA-dependent cAMP production was abolished only by pretreatment with the A_{2A} receptor antagonist CSC; 4) the dose-dependency of basolateral CPA stimulation of Na^+/H^+ exchange observed in this study is very similar to that reported for basolateral CPA stimulation of cAMP production (12); and 5) pharmacological increases in intracellular cAMP concentration by either forskolin or 8-bromo-cAMP resulted in a stimulation of Na^+/H^+ exchange similar to that of basolateral CPA treatment. Regarding this point, we also reported a stimulatory action of cAMP on Na^+/H^+ exchange instead of the more commonly observed inhibition (17) in the same A_6 clone (18) and in other cell types (30, 38–40). 6) The specific inhibition of PKA with H-89 (29, 30) completely blocked the stimulation of Na^+/H^+ exchange by basolateral CPA. Taken together, these findings support the view that CPA binding to A_{2A}-like receptors localized on the basolateral membrane stimulates Na^+/H^+ exchange via adenylate cyclase/PKA.

Apical CPA action on Na^+/H^+ exchange. A slight but significant inhibition of Na^+/H^+ exchange was observed on apical application of CPA to A_6 monolayers (Figs. 1 and 2). The hypothesis that this inhibition was due to the action of an apically located adenosine receptor related to the mammalian A_1 receptor is supported by the observation that apical CPA-dependent inhibition of Na^+/H^+ exchange activity was significantly reduced by the A_1 receptor-selective antagonist CPX (25).

Because our previous studies indicated that CPA interacting with the A_1 receptors induces an elevation of cytosolic calcium by release of intracellular stores (12), we examined the possibility that the inhibitory effect of apical CPA on Na^+/H^+ exchange activity could be mediated by activation of the intracellular Ca^{2+}/PKC system. The findings that both ionomycin and TPA inhibited Na^+/H^+ exchange activity and that the inhibition of PKC by calphostin C prevented the CPA effect suggest that apical CPA inhibition of Na^+/H^+ exchange activity is mediated, at least in part, through PKC activation.

Coupling between pH_i and the transepithelial Na^+ transport. The dual-control regulation of the Na^+/H^+ exchanger and the presence of two types of adenosine receptors

linked to distinct postreceptor mechanisms suggest that adenosine can exert different effects on pH_i depending on both the external adenosine concentration and the site of action. It has been well documented that pH_i seems to be a possible mediator coupling the rate of Na^+ transport across both the apical and basolateral membranes. Alterations in pH_i has been reported to directly influence apical Na^+ conductance and/or indirectly the basolateral Na^+/K^+-ATPase (13–16). The relationship between pH_i and Na^+ transport seemed to be reciprocal: an increase in H^+ concentration was reported to cause a decrease in transepithelial Na^+ transport. The current findings together with our previous observations that basolateral CPA increases Na^+ transepithelial transport via A_2 adenosine receptors (12) suggest that the intracellular signal by which adenosine stimulates transepithelial Na^+ transport could be cellular alkalinization via cAMP-dependent stimulation of Na^+/H^+ exchange. In line with this hypothesis are the results that we obtained in A_6 monolayers treated with the specific inhibitor of the Na^+/H^+ exchanger, HOE-694 (31).

In addition to decreasing the resting pH_i, HOE-694 significantly inhibited the basolateral stimulatory effect of CPA on transepithelial sodium transport. These results clearly demonstrate that changes in pH_i are able to modulate the transepithelial sodium transport in response to adenosine action and imply a role of the Na^+/H^+ exchanger as a second messenger.

In conclusion, we were able to demonstrate that in A_6 cell monolayers, adenosine can exert various effects on pH_i depending on the site of action. The basolateral addition of adenosine stimulated Na^+/H^+ exchange activity via an increase in cAMP/PKA, whereas apical adenosine addition inhibited the activity of the exchanger, probably via intracellular Ca^{2+}/PKC. These results together with those of previous reports showing that basolateral CPA stimulation of transepithelial Na^+ transport is mediated by activation of adenylate cyclase (12) imply that this stimulation of Na^+ transport may be mediated by the change in Na^+/H^+ exchanger activity.

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