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Effect of Adenosine on Na^+ and Cl^- Currents in A_6 Monolayers. Receptor Localization and Messenger Involvement

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Abstract. The effect of adenosine regulation on sodium and chloride transport was examined in cultured A_6 renal epithelial cells. Adenosine and its analogue N^6-cyclopentyladenosine (CPA) had different effects on short-circuit current (I_{sc}) depending on the side of addition. Basolateral CPA addition induced an approximately threefold increase of the I_{sc} that reached a maximum effect 20 min after addition and was completely inhibited by preincubation with either an A_2 selective antagonist, CSC, or the sodium channel blocker, amiloride. Apical CPA addition induced a biphasic I_{sc} response characterized by a rapid fourfold transient increase over its baseline followed by a decline and a plateau phase that were amiloride insensitive. The A_1 adenosine antagonist, CPX, completely prevented this response. This I_{sc} response to apical CPA was also strongly reduced in Cl^--free media and was significantly inhibited either by basolateral bumetanide or apical DPC preincubation. Only basolateral CPA addition was able to induce an increase in cAMP level. CPA, added to cells in suspension, caused a rapid rise in $[Ca^{2+}]_i$ that was antagonized by CPX, not affected by CSC and prevented by thapsigargin preincubation. These data suggest that basolateral CPA regulates active sodium transport via A_2 adenosine receptors stimulating adenylate cyclase while apical CPA regulates Cl^- secretion via A_1 receptor-mediated changes in $[Ca^{2+}]_i$.

Key words: Adenosine receptors — A6 cells — Chloride channels — Sodium transport — cAMP — Calcium

Introduction

As a potential autocrine or paracrine factor, adenosine has been demonstrated to be released by the kidney during ischemia and sodium loading and to modulate ion transport by activating specific membrane receptors and intracellular signaling pathways [29, 34]. In the kidney, as in other tissues, at least two adenosine receptors have been identified based on their affinity for various adenosine analogues and their effect on adenylate cyclase. The A_1 receptor has a high affinity (in nanomolar range) for adenosine and inhibits adenylate cyclase while stimulating phosphoinositide turnover and mobilizing $[Ca^{2+}]_i$. The A_2 receptor has a much lower affinity (in micromolar range) for adenosine and stimulates adenylate cyclase [19, 29]. Recently, a number of A_1 and A_2 receptor agonists and antagonists have been developed that are useful in the differentiation of A_1- from A_2-mediated events and in the functional characterization of its action and of the spatial location of its receptors.

The presence of both A_1 and A_2 adenosine receptors has been demonstrated in several renal cell lines derived from different nephron segments of various species [2, 15, 16, 24, 27, 35]. A_6 cells, derived from the kidney of *Xenopous laevis* [25], share many properties with principal cells of the mammalian renal collecting duct. When grown on permeable filter supports these cells form tight, polarized monolayers with high resistance, a spontaneous lumen negative potential difference and net apical to basolateral Na^+ flux [22]. Previous reports demonstrated that in addition to the amiloride-sensitive Na^+ channel current, a Cl^--dependent component of current was evoked by ADH [5, 33, 36]. Three types of Cl^- channels have been reported in the apical membrane of A_6 cells and at least one of these channels is regulated by changes in either cAMP or Ca^{2+} [17, 18, 20]. Owing to the high resistance of the paracellular pathway this homogenous monolayer presents a very useful model for the study of the functional linkage between apical and basolateral membranes during variations in Na^+ and Cl^- currents and its regulation by hormones and related agents.

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Although the presence of adenosine A_1 [13] and A_2 [15] receptors has been demonstrated in A_6 cells, their role in the regulation of ion transport and the signal transduction pathways involved is controversial. If both receptors are present, their respective physiological action on Na^+ and Cl^- transport is of physiological interest.

The present study was performed: (i) to examine the effect of adenosine on Na^+ and Cl^- ion transport properties (ii) to elucidate the signal transduction pathways by which adenosine regulates Na^+ and Cl^- transport and (iii) to determine the spatial distribution of the A_1 and A_2 receptors to the apical and/or basolateral cell surface. We demonstrate that A_6 cells contain both A_1 and A_2 adenosine receptors and that A_1 receptors are located on the apical surface and regulate apical Cl^- secretion via calcium while basolateral A_2 receptors regulate Na^+ transport via an increase in cAMP.

Materials and Methods

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 A6-2F3 cells at passage 99 and was selected for its high transepithelial resistance and for its responsiveness to aldosterone and antidiuretic hormones [32].

Cells were cultured in plastic culture flasks at 28°C in 5% CO_2 atmosphere in 0.8 × concentrated DMEM (Gibco) containing 25 mM $NaHCO_3$ and supplemented with 10% heat-inactivated fetal bovine serum (Flow) and 1% of a penicillin-streptomycin mix (Seromed) (final osmolality of 220–250 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 reported, with the exception of calcium measurements, cells were plated on permeant filter supports (Transwell, 0.4 μm pore size, 4.7 cm^2 Costar) previously coated with a thin layer of rat tail collagen (Biospa) according to published methods [32]. Experiments were generally performed 10 to 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. In these culture conditions A_6-C1 cells exhibit tight junctions and small apical microvilli as confirmed by electron microscopy studies (*data not shown*).

Measurement of Transepithelial Short-Circuit Current

Measurements of transepithelial current (μA/cm^2) and voltage (mV) were performed in a modified Ussing chamber [21] using an automatic voltage-clamp apparatus, which was connected to a one-channel recorder (Pharmacia LKB). The mucosal and serosal reservoirs contained 5 and 3 ml, respectively, of a Ringer solution of the following composition (in mM): 110 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgSO_4$, 1 KH_2PO_4, 5 glucose and 10 Hepes buffered with Tris to a final pH = 7.5. In some experiments, Cl^- was replaced isosmotically with gluconate and sulfate. Calomel voltage electrodes connected to the medium with thin polythylene tubing containing 3% agarose in Na medium, and inert platinum current electrodes were used.

Transepithelial resistance ($\Omega \times cm^2$) was calculated according to Ohm's law. During experiments the cell monolayers were kept in open-circuit configuration and the transepithelial potential difference (V_t) was monitored continuously. The short-circuit current (I_{sc}) was measured every 1 min by clamping V_t to 0 mV for 5 secs.

Cyclic AMP Determination

Intracellular cAMP levels were analyzed as previously reported [4]. Cell monolayers grown on filter inserts were placed in the A_6 Ringer described above and exposed to hormones for 10 min in the presence of 1 mM rolipram, a phosphodiesterase inhibitor that is not an adenosine receptor antagonist. To study the localization of adenosine receptors and the adenosine-mediated pathways, CPA and the A_1 antagonist CPX, were added to either the basolateral or to the apical side of the monolayers. The monolayers were rapidly rinsed twice with ice-cold assay buffer in mM): 50 TRIS/HCl, 16 2-mercaptoethanol, 8 theophylline, pH 7.4 and the monolayers immediately immersed in liquid nitrogen. The filter apparatus was stored at −20°C 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 eppendorf tube. Cells were disrupted by two 5-sec pulses with a probe sonicator (Branson), the sample neutralized with 10 μl 0.1 N NaOH and the filter plus cell debris removed by centrifugation at 14,000 rpm for 15 sec in an Eppendorf centrifuge. The cAMP concentration was determined on a 50 μl aliquot of the supernatant using the test kit from NEN-Dupont (Boston, MA) based on a competitive protein-binding assay [3].

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in cell suspension was measured using the Fura-2 fluorescent dye technique. Briefly, cells were detached by trypsinization and incubated for 60 min at 28°C with continuous shaking in growth medium plus 5 μM Fura-2/AM. After this loading period, the cells were washed three times and resuspended to a concentration of 8×10^5 cells/ml in Ringer medium containing (in mM): 101 NaCl, 5.4 KCl, 8 $NaHCO_3$, 1.4 $CaCl_2$, 1.7 $MgSO_4$, 0.9 NaH_2PO_4, 5 glucose, 1 HEPES (pH = 7.5). Fluorescence was measured with a dual excitation wavelength spectrofluorometer (Shimadzu RF 5000).

Samples were excited at 340 nm and 380 nm (bandwidth 3 nm) and emission was monitored at 510 nm (bandwidth 5 nm). The 340/380 ratio was converted to an actual $[Ca^{2+}]_i$ according to the calibration procedure described by Grynkiewitcz [12].

Materials

Fura-2/AM was from Molecular Probes (Eugene, OR). Thapsigargin and lonomycin were obtained from Calbiochem. All other substances were obtained from Sigma.

Data Analysis and Statistics

Data presented are expressed as mean ± SE. Statistical significance was assessed by *t*-test for paired and unpaired data. A value of $P < 0.05$ was accepted as significant.

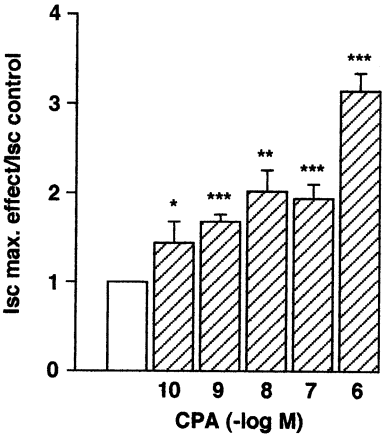

Fig. 1. Concentration-response of short-circuit current (I_{sc}) to CPA. Relative I_{sc} [$I_{sc}(t = 20$ min.)/$I_{sc}(t = 0)$] is reported on the ordinate. A_6 cell monolayers were placed in a modified Ussing chamber and bathed in amphibian Ringer solution. The preparations were maintained in open-circuit configuration during the recordings and the transepithelial short-circuit current was measured for 5 sec every 60 sec. Values are mean ± SE of 5 monolayers at each concentration of CPA added to both bathing media. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, (paired *t*-test).

ABBREVIATIONS

CGS-21680:	2-[4-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamido adenosine hydrochloride
DPC:	diphenylamine carboxylic acid
CPA:	N^6-cyclopentyladenosine
CPX:	1,3-dipropyl-8-cyclopentylxanthine
CSC:	8-(3-Chlorostyryl)caffeine
IP_3:	inositol-1,4,5-trisphosphate

Results

The effect of adenosine was studied in A_6 (clone A_6-C1) cells cultured on permeable support filters, as reported in Materials and Methods, in media containing fetal bovine serum but without supplemental aldosterone. After 10–14 days growth under these conditions, A_6 monolayers displayed a high transepithelial resistance (9,850 ± 467 $\Omega \times cm^2$, $n = 40$) and substantial short-circuit current (5–10 $\mu A/cm^2$). To study the effect of adenosine on the physiological parameters of the A_6 cell monolayers we used N^6-cyclopentyladenosine (CPA), a metabolically stable adenosine analogue little taken up by cells, that binds both adenosine receptors subtypes but with a higher affinity for the A_1 receptor [31].

EFFECT OF CPA ON I_{sc}

As the extracellular free adenosine concentration in the kidney ranges from 10^{-7} to 10^{-6} M (1), we chose to first measure the concentration dependency of CPA from 10^{-10} to 10^{-6} M. Figure 1 shows the dose-response of

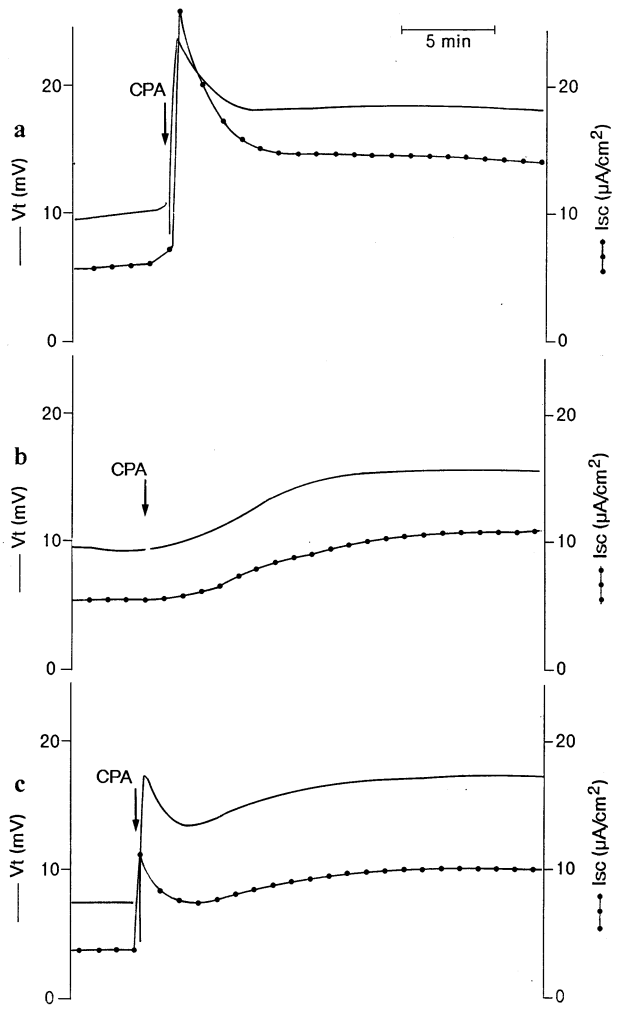

Fig. 2. Representative traces depicting the effect of 10^{-6} M CPA on short-circuit current (I_{sc}) and potential difference (V_t) in A_6 monolayers. Monolayers were treated as in Fig. 1. After an initial period in which I_{sc} was allowed to stabilize, CPA was added to the apical side of the monolayer (*a*), to the basolateral side (*b*) or to both sides (*c*).

CPA added to both sides of the monolayer on I_{sc} in A_6 epithelial monolayers placed in a Ussing-type chamber. The data are displayed as the maximum I_{sc} after 20-min incubation divided by the initial (control) value. An increase in I_{sc} with increasing CPA concentration was observed. We then analyzed the cell surface polarity of the effect of CPA on I_{sc} at the maximum concentration, 10^{-6} M. Figure 2 shows typical time courses of I_{sc} after addition of CPA to A_6 epithelial monolayers. CPA applied to the apical surface (Fig. 2*a*) induced a biphasic increase of I_{sc}: a transient increase of I_{sc} that peaked 60 sec after addition of CPA followed by a rapidly declining I_{sc} that reached a stable level that was higher than the initial, control value by 20 min. The basolateral addition of CPA (Fig. 2*b*) produced only a late and sustained increase in I_{sc} which reached a maximum effect 20 min

Table 1. CPA effect on the short-circuit current in A_6 monolayers

Experimental condition of 10^{-6} M CPA addition	n	I_{sc} after CPA/I_{sc} baseline	
		Transient early effect (1.5 min after CPA)	Late effect (20 min after CPA)
Apical	9	3.93 ± 0.46	2.70 ± 0.12
Basolateral	9	1.04 ± 0.02	2.72 ± 0.23
Apical CPA after CPX preincubation	3	1.05 ± 0.02[b]	1.08 ± 0.06[b]
Basolateral CPA after CPX preincubation	5	1.02 ± 0.03[a]	2.02 ± 0.24[a]

Values are mean ± SE CPX (10^{-7} M) was added 15 min before the addition of CPA both in the apical and in the basolateral perfusion solutions. Significant difference of CPX effect on observed CPA stimulation: [a]NS, [b]$P < 0.001$ (unpaired *t*-test).

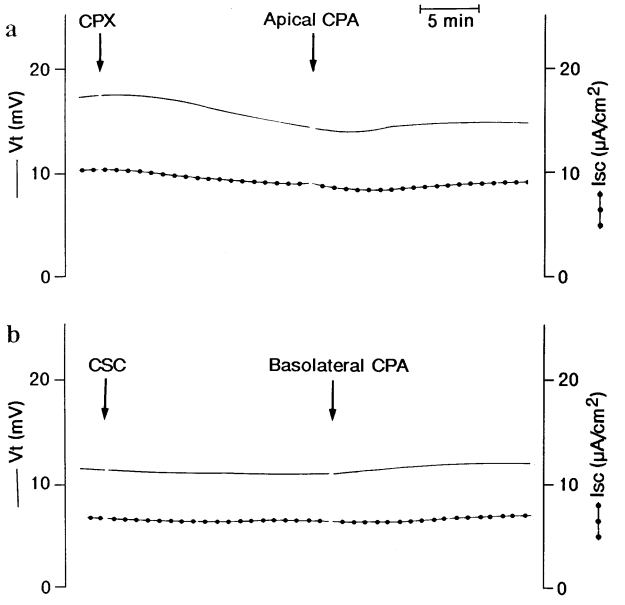

Fig. 3. Time course of short-circuit current response to 10^{-6} M CPA addition: (**a**) to the apical side of the monolayer after a 15-min preincubation of 10^{-7} M CPX (A_1 selective antagonist) to the same side of the monolayer; (*b*) to the basolateral side of the monolayer after a 10-min basolateral preincubation of 10^{-6} M CSC (A_2 selective antagonist).

after the addition of the hormone. The effect of CPA addition to both sides of the monolayer (Fig. 2*c*) paralleled the combined apical and basolateral effect of CPA. In Table 1, the I_{sc} results obtained in experiments carried out as in Fig. 2 are summarized. As can be seen, apical CPA significantly increased both the transient and late I_{sc} response in respect to the control ($P < 0.001$ for both), while basolateral CPA significantly increased I_{sc} only in the late response ($P < 0.001$). Interestingly, CPA added to both sides of the monolayer induced an early, transient I_{sc} increase identical to that induced by apical CPA alone (4.01 ± 0.26, $n = 6$, *vs.* 3.93 ± 0.45, $n = 9$, respectively. *ns*) and a late increase of I_{sc} (3.53 ± 0.19, $n = 6$) that was significantly higher than that induced by either apical CPA (2.70 ± 0.12, $P < 0.01$, $n = 9$) or basolateral CPA alone (2.72 ± 0.23, $P < 0.05$, $n = 9$); suggesting a combination of the two different effects induced by CPA. Natural adenosine when added to the A_6 monolayers produced the same qualitative effects obtained with CPA (*data not shown*). Although CPA was less potent than adenosine, CPA gave results more homogenous probably due to its lower rate of metabolic breakdown.

To determine if these responses to CPA were via receptor-mediated mechanisms and if the adenosine A_1 and A_2 receptors might have an asymmetrical cell surface localization, we examined the effect of the A_1 selective receptor antagonist, CPX [31], on CPA action. In all the A_6 monolayers examined, the baseline I_{sc} and V_t were not significantly altered by addition of CPX alone (*data not shown*). CPA was added to either the apical or basolateral solution after a 15-min preincubation of CPX to the same side of the monolayer. While basolateral CPX only slightly, but not significantly, inhibited the basolateral CPA effect (Table 1), apical CPX completely abolished the biphasic response to apical CPA (Fig. 3*a* and Table 1). Thus, it is possible that CPA added to the apical side causes the rapid increase of I_{sc} by interacting with specific apically located A_1 adenosine receptors whereas CPA added to the basolateral side induced the late increase of I_{sc} mainly by interacting with A_2 receptors basolaterally located. Further support for this notion comes from the observation that the A_2 selective antagonist [14], CSC (10^{-6} M), applied on the basolateral side of the monolayer for 10 min completely prevented the late effect of basolateral CPA (Fig. 3*b*) while not significantly altering the baseline I_{sc} (6.67 ± 0.92 I_{sc} in basal conditions and 7.5 ± 1.23 I_{sc} after 15 min of preincubation, $n = 4$).

The addition of the apical Na^+ channel blocker, amiloride (10^{-5} M), caused an immediate and expected fall in both transepithelial V_t and I_{sc} (Fig. 4), revealing that a substantial part of the I_{sc} in basal conditions corresponded to electrogenic Na^+ transport even if the A_6 cells were cultured without aldosterone treatment. The subsequent addition of CPA to the basolateral side no longer elicited a response of the electrical parameters, suggesting that the late increase of basolateral CPA-induced I_{sc} observed in the absence of amiloride could correspond to the known cAMP-mediated effect on the amiloride-sensitive Na^+ channels. The subsequent apical addition of CPA still elicited a large increase of the I_{sc} suggesting that the transport of ions other than sodium was stimulated. To determine whether the amiloride-insensitive I_{sc} induced by apical addition of CPA was due to electrogenic Cl^- secretion, we first analyzed the

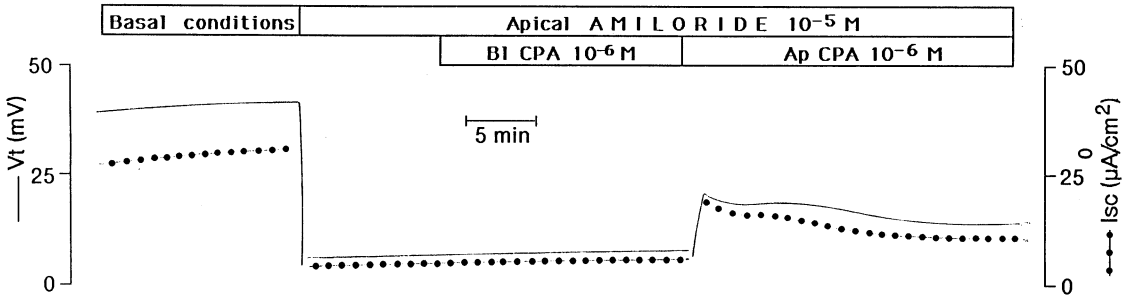

Fig. 4. A representative trace depicting the response of the amiloride-insensitive component of I_{sc} to 10^{-6} M CPA added to the apical side of the monolayer. After an initial period in which the I_{sc} was allowed to stabilize, amiloride (10^{-5} M) was added to the apical compartment 10 min before the addition of CPA (10^{-6} M) to the basolateral compartment. After 20 min of incubation in the above conditions CPA was added to the apical compartment.

effect of the preincubation of bumetanide, an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter, on the response of I_{sc} to CPA. The entry of Cl^- across the basolateral membrane of A_6 monolayers has been demonstrated to occur via the $Na^+/K^+/2Cl^-$ cotransporter [9, 36]. The addition of 0.1 mM bumetanide to the solution bathing the basal surface of the A_6 monolayers did not alter basal I_{sc} (2.22 ± 0.35 *vs.* 2.63 ± 0.35 μA/cm^2 before and after bumetanide addition, respectively) while significantly inhibiting the apical CPA-induced transient increase (Table 2). These experiments indicate that the $Na^+/K^+/2Cl^-$ cotransporter plays an important role in net basal-to-apical Cl^- flux. The response to apical CPA was also significantly inhibited after the addition of diphenylamine carboxylic acid (DPC), an inhibitor of some epithelial Cl^- channels [7] to the apical perfusion solution (Table 2). Further support for the hypothesis that apical CPA-induced Cl^- secretion was derived from experiments in which the response to apical CPA was examined during perfusion of both sides of the A_6 cell monolayers with Cl^--free Ringer solution. Substitution of chloride by gluconate reduced the baseline I_{sc} by 51.2 ± 10.2% ($n = 3$) and strongly inhibited the early increase of I_{sc} in response to apical CPA (transient early increase in I_{sc} after apical CPA/I_{sc} baseline = 1.22 ± 0.02).

Table 2. Effects of apical CPA on A_6 monolayers pretreated with Bumetanide or DPC

Experimental condition	*n*	I_{sc} after CPA/I_{sc} baseline	
		Transient early effect (1.5 min after CPA)	Late effect (20 min after CPA)
Apical CPA	10	3.81 ± 0.43	2.66 ± 0.11
CPA after bumetanide	5	1.71 ± 0.14[a]	1.84 ± 0.09[a]
CPA after DPC	5	1.58 ± 0.13[a]	1.76 ± 0.17[b]

Values are mean ± SE. Bumetanide (10^{-4} M) was added to the basolateral side of the A_6 monolayers 10 min before addition of CPA (10^{-6} M) to the apical solution. 10^{-4} M DPC was added to the apical side 5 min before the addition of CPA to the same side. Significant difference from apical CPA values: [a]$P < 0.01$, [b]$P < 0.001$ (unpaired *t*-test).

Intracellular Calcium Measurements

To analyze the signaling pathways that may be coupled to the A_1 and A_2 adenosine receptors we first studied the effect of CPA on $[Ca^{2+}]_i$ in suspended A_6 cells. Figure 5 (*a*) shows that (10^{-6} M) CPA added in a Ca^{2+}-containing medium caused an increase in $[Ca^{2+}]_i$ of 135 ± 25% over the baseline value of 65.57 ± 12.52 nM, $n = 8$. The CPA-induced Ca^{2+} mobilization was completely blocked by the selective A_1 receptor antagonist CPX (10^{-7} M) (*b*), but not by the selective A_2 antagonist CSC (*data not shown*). Furthermore, the A_2 adenosine agonist, CGS 21680 [31], was completely ineffective in elevating $[Ca^{2+}]_i$. We obtained the same results in parallel experiments carried out in Ca^{2+}-free medium 0.1 mM EGTA. Thapsigargin is reported to increase Ca^{2+} ions by inhibiting Ca^{2+} sequestration by the IP_3-sensitive stores [30]. Exposure of A_6 cells to 2 μM thapsigargin in Ca^{2+}-free medium increased $[Ca^{2+}]_i$ from 60.5 ± 15 to 222.5 ± 17.5 nM (n = 4) and completely abolished the CPA induced Ca^{2+} response (*data not shown*). These studies suggest that A_6 cells express an A_1 receptor for adenosine that is coupled to the release of intracellular calcium and the main source of calcium in the CPA induced response was via the release of intracellular stores. The possibility that an increase in $[Ca^{2+}]_i$ was sufficient to activate chloride secretion is supported by the observation that either 5 μM A23187 (Fig. 6), 2 μM ionomycin or 2 μM thapsigargin (*data not shown*) induced an increase in I_{sc} similar to that induced by apical CPA.

Measurements of Intracellular cAMP

In Fig. 7 are reported the alterations of the intracellular messenger cAMP level induced by various concentra-

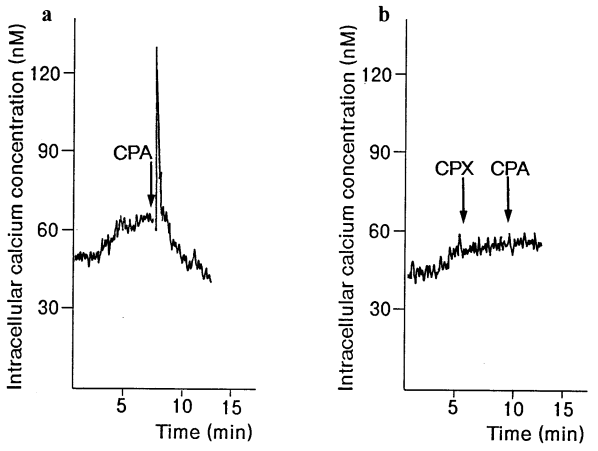

Fig. 5. Effect of CPA on intracellular calcium. Fura-2-loaded A_6 cells were incubated in Ca^{2+}-containing solution (1 mM). Where indicated CPA (10^{-6} M) and/or the A_1 selective antagonist CPX (10^{-7} M) were added. Results shown are representative of at least three determinations for each condition.

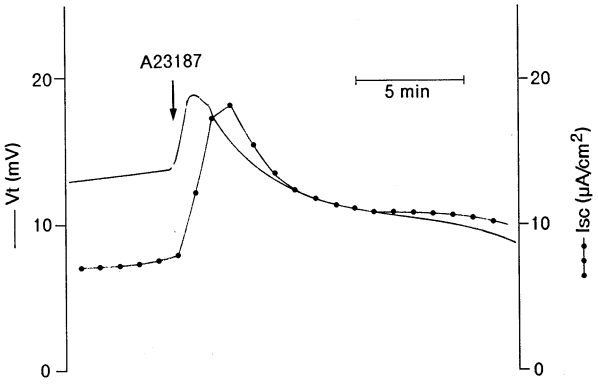

Fig. 6. Representative time course of short-circuit current responses to the ionophore A23187 (10^{-6} M) added to the apical side of the monolayer. The same response was found in four monolayers examined.

tions of CPA (10^{-8} to 10^{-5} M) added to either solution bathing A_6 cells monolayers. As shown, CPA added to the apical side of the monolayer had no effect on the cAMP content whereas basolateral CPA caused an increase in cAMP level in a dose-dependent manner. The preincubation for 10 min with the A_1 selective antagonist CPX did not prevent the increase in cAMP level induced by basolateral CPA (Fig. 8) confirming the hypothesis that basolateral CPA activates adenylate cyclase by interacting with A_2 receptor localized on the basolateral membrane.

Discussion

In the current study we confirm and extend previous studies that demonstrated that in A_6 cell monolayers

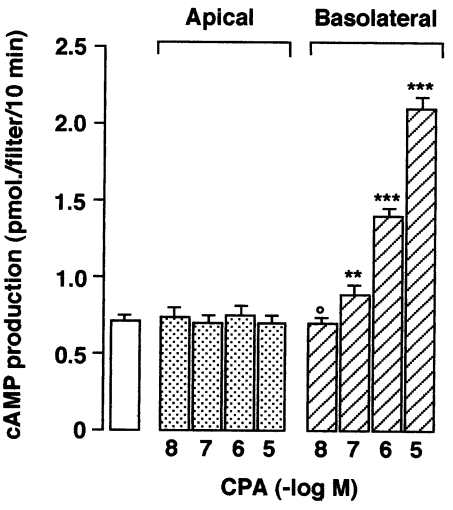

Fig. 7. Concentration effect of CPA applied apically or basolaterally on the generation of cAMP. After 10 min preincubation with 10 μM of the phosphodiesterase inhibitor, rolipram, A_6 cell monolayers were exposed to varying concentrations of CPA for 20 min. Levels of cAMP were determined as described in Materials and Methods. Values are mean ± SE for three experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, °not significant (paired *t*-test).

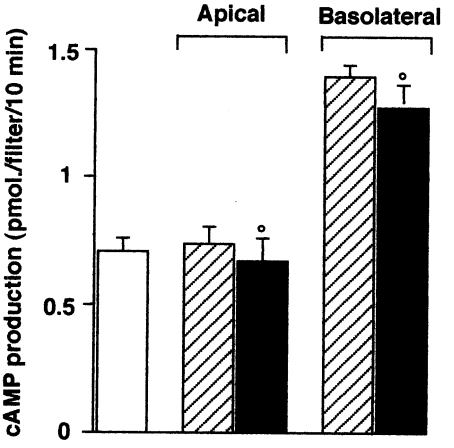

Fig. 8. Effect of preincubation with the A_1 selective antagonist CPX on the cAMP generation induced by apical or basolateral CPA. CPX (10^{-7} M) was added 15 min before the addition of CPA (10^{-6} M) to either the apical or to the basolateral side of the A_6 monolayer, respectively. Striped bars represent CPA alone and solid bars represent pretreatment with CPX before CPA. Values are mean ± SE for 3 experiments. °not significant (paired *t*-test).

adenosine increases active sodium transport by stimulating adenylate cyclase [15]. The functional localization of the adenosine A_1 and A_2 receptors was assessed by spatially stimulating the A_6 cell monolayers with CPA, a metabolically stable analogue of adenosine, which binds both adenosine receptors with a higher affinity for the A_1. The results of the present study demonstrate that two

types of adenosine receptors linked to different transduction pathways and to different ion transport systems reside on different cell surfaces of these cells. We found that CPA when added to the basolateral side increases Na^+ transport by interacting with basolaterally located A_2 receptors whereas when added to the apical side CPA induced an increase in Cl^- conductance by acting on A_1 receptors.

Three lines of evidence support the conclusion that a topographic separation of the adenosine A_1 and A_2 receptors exist in A_6 epithelia. First: Apical CPA induced, in all monolayers examined, a biphasic effect on I_{sc} composed of an immediate but transient increase of I_{sc} followed by a plateau phase, whereas the time course of the I_{sc} response to basolateral CPA was monophasic with a maximum effect after 20 min. Second: In the presence of the apical Na^+ channel blocker, amiloride, basolateral CPA no longer increased I_{sc} whereas apical CPA was still able to induce I_{sc} with the usual time course suggesting that the transport of ions other than sodium was stimulated. Third and most convincing: Apical preincubation with CPX, an A_1 receptor antagonist, abolished the biphasic response to apical CPA whereas basolateral CPX preincubation did not affect the late increase of I_{sc} usually induced by basolateral CPA. Further support to this hypothesis came from the finding that the late effect of basolateral CPA was completely prevented by basolateral preincubation with CSC, an A_2 selective antagonist.

Coexpression of A_1 and A_2 adenosine receptors on the same cell has already been reported for rabbit cortical collecting duct cells [2], rat inner medullary collecting duct cells [35] and, recently, a human cortical collecting duct cell line [24]. Due to the low transepithelial resistance of these epithelia, it was difficult in those studies to clearly analyze the spatial location of the adenosine receptors. In the present study, we were able to grow monolayers that developed a very high transepithelial resistance ($9{,}850 \pm 467\ \Omega \times cm^2$) that permitted us to functionally separate spatially the apical from basolateral located processes. The differences we observed between mucosal and serosal responses to CPA reflect differences in post receptor mechanisms regulating sodium and chloride conductances.

Basolateral CPA and Sodium Transport Regulation

We found that only basolateral CPA exposure elicited an increase in both amiloride-sensitive I_{sc} (Fig. 4) and cAMP content (Fig. 7). The fact that the regulation of cAMP production correlates with the regulation of sodium transport is compatible with the hypothesis that the cAMP pathway is involved in the transduction of the A_2 effect on sodium transport. In line with these data, it has been previously shown that cAMP can stimulate transepithelial sodium transport across A_6 cell epithelia [33].

Apical CPA and Chloride Transport Regulation

The biphasic apical CPA stimulation of I_{sc} was characterized by a rapid fourfold transient increase of I_{sc} over its baseline, followed by a decline and a plateau phase. The observation that the A_1 adenosine antagonist CPX completely prevented both the transient increase and the plateau phase confirms the hypothesis that CPA stimulated I_{sc} via the apically located A_1 adenosine receptors. That electrogenic Cl^- secretion represents one of the major components of the transient increase induced by apical CPA is supported the following observations: (i) The biphasic stimulation of I_{sc} was not prevented by amiloride addition; (ii) Both basolateral bumetanide, an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter, and apical addition of DPC, a blocker of Cl^- channels in numerous Cl^- transporting epithelia [7], reduced by more than 50% the transient increase of I_{sc} induced by apical CPA and (iii) Substitution of Cl^- by gluconate inhibited the biphasic response to apical CPA.

Chloride channels have been identified in the apical membrane of principal cells in the cortical collecting duct [6, 11], in Madin-Darby canine kidney cells [28] and in A_6 cells [17, 33] which are known to share many properties with principal cells of the mammalian renal collecting duct. In the A_6 cells three types of Cl^- channels have been reported in the apical membrane and a least one of these (the small single channel conductance, 3pS) is regulated by changes in $[Ca^{2+}]_i$ [18]. We found that CPA was able to induce a rapid rise in $[Ca^{2+}]_i$ with a peak at 30 sec either in the presence or in the absence of external calcium. Thapsigargin completely abolished the response to CPA, supporting the notion that CPA released Ca^{2+} from the same intracellular compartment that was depleted by thapsigargin: the IP_3-sensitive stores. Furthermore, because the increase in cytosolic calcium in response to CPA was blocked by low concentrations of the A_1 selective antagonist CPX, but not by the A_2 antagonist CSC, we conclude that this CPA effect was mediated via A_1 receptors. This CPA-dependent stimulation of $[Ca^{2+}]_i$ together with the observation that both calcium ionophores (ionomycin and A23187) or 2 μM thapsigargin caused a transient increase of I_{sc} similar to that observed in response to apical CPA, suggests that the A_1 receptor-mediated adenosine action on Cl^- conductance was due at least in part to its effect on $[Ca^{2+}]_i$.

Our finding that adenosine analogues cause both a $[Ca^{2+}]_i$ increase and Cl^- channel activation by interacting with the A_1 receptors is consistent with the results reported recently in airway epithelial cells [26]. Also in the rabbit cortical collecting duct cell line, RCCT-28A, stimulation of adenosine A_1 receptors induces Cl^- secretion through a complex pathway involving phospholipase C, Protein Kinase C and a G protein [27]. Although our experiments cannot establish the full intracellular

mechanism responsible for the spike of calcium and the consequent increase in Cl^- conductance in response to apical CPA, we can hypothesize that also in the A_6 cell line adenosine may be initiating the turnover of inositol phospholipids leading to the increased production of inositol trisphosphate and the release of intracellular calcium stores.

Although a direct calcium-dependent regulation of the apical chloride channels is possible, it might be that a basolateral calcium activated K^+ channel also plays a role in the linkage between agonist action and chloride movement as proposed by Petersen and Maruyama [23]. In fact, a K^+ conductance activated via elevation of cytosolic Ca^{2+} has recently been identified on the basolateral membranes of the A_6 monolayers [8].

In conclusion, the results of the present study provide evidence for coexpression of A_1 and A_2 adenosine receptors on different cell surface membranes of the same cell. The presence of the two types of adenosine receptors were linked to distinct postreceptor mechanisms modulating different transport of ions. This suggests that adenosine can exert various effects depending not only on the external concentration but also on the site of action. In addition to the classical mechanism involving adenylate cyclase induced by basolateral A_2 adenosine receptors our data indicate that in A_6 cells, as in other epithelia, adenosine can regulate chloride secretion. This regulation of chloride secretion is mediated by A_1 adenosine receptors located only on the apical cell surface and transduced by calcium released from intracellular stores. As has been reported in rabbit cortical collecting duct cells [27], this chloride secretion may serve to restore cell volume during renal ischemia and sodium loading. The A_6 cells provide a useful model for the further analysis of the interaction of apical- and basolateral-located regulatory mechanisms and the importance that spatial separation of receptor subtypes might play in their coordinated function.

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