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Activation of A_3 Adenosine Receptor Induces Calcium Entry and Chloride Secretion in A_6 Cells

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Abstract. We have previously demonstrated that in A_6 renal epithelial cells, a commonly used model of the mammalian distal section of the nephron, adenosine A_1 and A_{2A} receptor activation modulates sodium and chloride transport and intracellular pH (Casavola et al., 1997). Here we show that apical addition of the A_3 receptor-selective agonist, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-methyluronamide (Cl-IB-MECA) stimulated a chloride secretion that was mediated by calcium- and cAMP-regulated channels. Moreover, in single cell measurements using the fluorescent dye Fura 2-AM, Cl-IB-MECA caused an increase in Ca^{2+} influx. The agonist-induced rise in $[Ca^{2+}]_i$ was significantly inhibited by the selective adenosine A_3 receptor antagonists, 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS 1523) and 3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191) but not by antagonists of either A_1 or A_2 receptors supporting the hypothesis that Cl-IB-MECA increases $[Ca^{2+}]_i$ by interacting exclusively with A_3 receptors. Cl-IB-MECA-elicited Ca^{2+} entry was not significantly inhibited by pertussis toxin pretreatment while being stimulated by cholera toxin preincubation or by raising cellular cAMP levels with forskolin or rolipram. Preincubation with the protein kinase A inhibitor, H89, blunted the Cl-IB-MECA-elicited $[Ca^{2+}]_i$ response. Moreover, Cl-IB-MECA elicited an increase in cAMP production that was inhibited only by an A_3 receptor antagonist. Altogether, these data suggest that in A_6 cells a G_s/protein kinase A pathway is involved in the A_3 receptor-dependent increase in calcium entry.

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Key words: A_3 receptor — Cl-IB-MECA — A_6 cells — Renal — Chloride — Calcium

Introduction

The ability of adenosine to bind to different receptor subtypes and activate different effector systems is believed to account for its pleiotropic actions in renal cells. Adenosine receptors are G protein-coupled receptors and have been classified into A_1, A_{2A}, A_{2B} and A_3 receptor subtypes on the basis of pharmacological studies (Fredholm et al., 1994). The A_1 and A_3 receptors inhibit adenylate cyclase by G_i protein and/or activate phospholipase C leading to an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). The A_2 receptors, divided into A_{2A} and A_{2B} are linked to a G_s and stimulate adenylate cyclase (Palmer & Stiles, 1995). Adenosine interacting with the A_3 receptors has been shown to have opposite effects (Jacobson, 1998): at low concentrations (nM) A_3 agonists are cytoprotective, whereas at high concentrations (>10 μM) they induce apoptosis in a variety of cell types (Kohno et al., 1996). A_3 receptor activation is cerebroprotective upon chronic agonist administration (von Lubitz et al., 1994) and plays a crucial role in mediating preconditioning protection improving metabolic tolerance to ischemia in the heart (Strickler, Jacobson & Liang, 1996).

It is known that endogenous adenosine influences renal electrolyte transport by regulating a variety of plasma membrane ion channels and transporters (McCoy et al., 1993; Friedlander & Amiel, 1995). Moreover, adenosine could play an important role as a modulator of hormonally regulated solute and water transport by regulating cAMP production and intracellular calcium levels. Ligand binding and *in situ* hybridization studies together

with genetic analysis have demonstrated the presence of A_1 and A_{2A}, A_{2B} receptors in the kidney (Weaver & Reppert, 1992; Kreinsberg, Silldorff & Pallone, 1997). Further, the physiological and pharmacological properties of these receptors have been studied in several renal cell lines derived from different nephron segments of various species (Lang et al., 1985; Arend et al., 1987; Le Vier, McCoy & Spielman, 1992; Schweibert et al., 1992; Hayslett et al. 1995; Hoenderop et al., 1998).

While the presence of A_3 adenosine receptors in the kidney has been demonstrated (Zhou et al., 1992), to date there are no studies regarding the presence and functional characteristics of these receptors in renal cell lines. We recently demonstrated that A_6 cells, a renal cell line commonly used as a model of the mammalian collecting duct principal cells, have a polarized distribution of A_1 and A_{2A} adenosine receptors. These receptors are implicated in the regulation of intracellular pH and the subsequent action on transepithelial sodium transport (Casavola et al., 1997). The aim of the present study was to demonstrate the presence of A_3 receptors and investigate the details of their signal transduction and their physiological role in A_6 cells utilizing a selective A_3 agonist, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-methyluronamide (Cl-IB-MECA: Jacobson et al., 1995) in conjunction with the specific A_3 antagonists, 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS 1523: Li et al., 1998) and 3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191: Jiang et al., 1996).

We report here that Cl-IB-MECA induces an increase in cytoplasmic calcium that was prevented by a nominally calcium-free external medium. This influx was sensitive to pretreatment with A_3 but not with A_1 or A_2 receptor antagonists. The action of Cl-IB-MECA was inhibited by H-89, a protein kinase A (PKA) inhibitor (Chijiwa et al., 1990), and potentiated by increasing intracellular cAMP by either forskolin or rolipram preincubation, suggesting a central role of cAMP/PKA in A_3 receptor regulation of intracellular calcium in A_6 cells. Moreover, using measurements of transepithelial short-circuit current (I_{SC}) in polarized monolayers, we have demonstrated that Cl-IB-MECA only when added to the apical side induces chloride secretion, activating both calcium and cAMP-dependent Cl^- conductances.

Materials and Methods

Cell Culture

Experiments were performed with A_6 cells from the A_6-Cl 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 for its responsiveness to aldosterone and antidiuretic hormone (Verrey, 1994). 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 230–250 mOsmol). 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 the cAMP levels and transepithelial short-circuit current measurements, cells were plated on permeant filter supports (Transwell 0.4 μm pore size, Costar, Cambridge, MA) previously coated with a thin layer of rat tail collagen (Biospa) according to published methods (Casavola et al., 1996). Experiments were generally performed 10 to 15 days after seeding and the monolayers were fed three times per week. The medium was always changed the day before the start of an experiment.

Adenosine Agonists and Antagonists Utilized

To distinguish between the involvement of the putative adenosine receptor subtypes in $[Ca^{2+}]_i$ increase and cAMP generation in A_6 cells, we utilized various adenosine agonists and antagonists, with the following agonist K_i values (in nM) reported for binding in various mammalian tissues (for review *see* Daly & Jacobson, 1995; Jacobson et al., 1995): CPA: A_1 = 0.6, A_{2A} = 460; A_3 = 240, DPMA: A_1 = 140, A_{2A} = 4.4, A_3 = 3600, NECA: A_1 = 6.3, A_{2A} = 10, A_{2B} = 1900, A_3 = 110. Cl-IB-MECA is 2500-fold selective for the rat A_3 *vs.* A_1 receptor and 1400-fold selective *vs.* the rat A_{2A} receptor (Jacobson et al., 1995).

The antagonists used have the following K_i (values in nM) in adenosine receptor binding; MRS 1191: rat A_1 receptor (rA_1) = 40,100, rA_{2A} > 100,000; rA_3 = 1420; human A_{2B} (hA_{2B}) > 30,000; hA_3 = 31,4; MRS 1523: rA_1 = 1560; rA_{2A} = 2100; rA_3 = 113; hA_{2B} > 25,000; hA_3 = 18.9; XAC: rA_1 = 1.2; rA_{2A} = 63; rA_3 = 29,000; hA_{2B} = 12.3; hA_3 = 71 (Ji & Jacobson, 1999; Li et al., 1999).

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

Cells were seeded at low density on glass coverslips and used the following day for microspectrofluorimetric measurements of cytoplasmic $[Ca^{2+}]$ with the dye Fura 2-AM (Grynkiewicz, Poenie & Tsien, 1985). Cells were loaded in tissue culture medium with Fura 2-AM (5 μM) and returned to the incubator for 60 min. Coverslips with dye-loaded cells were mounted into a chamber placed on the stage of an inverted microscope (Zeiss IM 35) and perfused at 25°C using a gravity-driven system at a rate of 1.5–2 ml/min. Emitted fluorescence from a single cell was measured in response to alternate pulses of excitation light (5 msec duration) at 340 and 380 nm using a computer controlled four-place sliding filter holder manufactured in-house. The emitted fluorescence (510 nm) was focused on a photomultiplier tube, amplified digitally, converted and sampled on an IBM-compatible computer. All measurements were automatically corrected for background. The ratio of emitted light from the two excitation wavelengths (340/380) of Fura-2 provide a measure of ionized cytoplasmic $[Ca^{2+}]_i$. Cytosolic free calcium concentration was calculated according to the formula of Grynkiewicz et al. (1985). The composition of the Ringer solution used in these experiments was (in mM): NaCl 101.4, $MgSO_4$ 0.5, KCl 5.4, $NaHCO_3$ 8, NaH_2PO_4 0.9, Hepes 1, glucose 5, $CaCl_2$ 1.4 (pH = 7.5). Nominally calcium-free Ringer was obtained by removing the $CaCl_2$ and adding 10^{-5} M EGTA from the above Ringer.

Measurements of Transepithelial Short-Circuit Current

Measurements of transepithelial potential difference (mV) and short-circuit current ($\mu A/cm^2$) were performed in a modified chamber according to published methods (Casavola et al., 1996). Transepithelial resistance (Ωxcm^2) was calculated according to Ohm's law. The electrical parameters were measured at room temperature in the following Ringer solution (in mM): NaCl 110, $MgSO_4$ 0.5, KCl 3, KH_2PO_4 1, Hepes 10, Glucose 5, $CaCl_2$ 1 (pH = 7.5). In some experiments Cl^- was replaced iso-osmotically with gluconate.

Cyclic AMP Determination

Intracellular cAMP levels were analyzed as previously reported (Casavola et al., 1996). Cell monolayers grown on filter inserts were placed in the A_6 Ringer solution described above and exposed to agents for 15 min in the presence of 1 mM rolipram, a phosphodiesterase inhibitor that is not an adenosine receptor antagonist. When utilized, the adenosine antagonists were added 5 min before the addition of agonist. 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°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 N HCl in an Eppendorf tube. Cells were disrupted by two 5-sec pulses with a probe sonicator (Branson), the sample neutralized with 10 μl of 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.

Materials

FURA2-AM, BAPTA-AM, Forskolin, H-89, pertussis toxin and cholera toxin were purchased from Calbiochem, [^{3}H]inositol was purchased from Amersham, Great Britain, U73122 and U73343 from SIGMA. Cl-IB-MECA was provided by SRI (Menlo Park, CA) as a part of the National Institute of Mental Health's Chemical Synthesis Program. XAC, NECA, and N-0840 were purchased from RBI. MRS 1523 and MRS 1191 were synthesized as described (Jiang et al., 1996; Li et al., 1998). ZM241385 was from Tocris Cookson (Ballwin, MO). SCH58261 was the kind gift of Dr. Ennio Ongini (Schering Plough SPA, Milan, Italy).

Data Analysis and Statistics

Data are expressed as mean ± SE. Statistical comparisons were made using the paired and unpaired data Student's t tests, and $P < 0.05$ indicated a statistical difference. The percent of the change in Cl-IB-MECA induced calcium response by different pharmacological agents is calculated as the change in the Cl-IB-MECA-dependent ΔF (Fmaximal effect − Fbaseline) before and after treatment.

Abbreviations

AVP, Arginine Vasopressin; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-methyluronamide; CPA, N^6-cyclopentyladenosine; DPMA, N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methyl-phenyl)ethyl]adenosine; FSK, forskolin; MRS 1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethylnyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; SCH 58261, {7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]1,2,4-triazolo[1,5c]pyrimine}; ZM 241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)-phenol; XAC, xanthine amine congener, (8-[4-[[[[(2-aminoethyl)-amino] carbonyl]methyl]oxy]phenyl]1,3-dipropylxanthine); MRS 1523, 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; NECA, adenosine 5′-ethyluronamide.

Results

Effect of Cl-IB-MECA on I_{sc}

A_6 cells, a cell line derived from the distal part of the nephron of the toad (*Xenopus laevis*) are known to have transporters for both electrogenic sodium uptake and for electrogenic chloride secretion. Patch-clamp experiments in A_6 cells demonstrated two types of apical Cl^- channels which are controlled by calcium and/or cAMP (Marunaka & Eaton, 1990). In addition, Ling et al. (1997) have demonstrated the existence of the chloride channel CFTR on the apical membrane of A_6 cells.

We have previously reported that A_6 cells form a high transepithelial resistance, ion transporting monolayer when grown on permeable support and can generate a short-circuit current (I_{sc}) modulated by A_1 and A_{2A} adenosine receptor activation (Casavola et al., 1996). Figure 1*A* shows that only apical application of the A_3 selective agonist, Cl-IB-MECA (Jacobson et al., 1995), elicited a transient increase of I_{sc}, after about 2 min, followed by a sustained plateau that was higher than the initial control value for at least 15 min and that this induction by Cl-IB-MECA of both the transient peak and the plateau phase were significantly inhibited by the preincubation of the monolayer with 10 nM of the specific A_3 receptor antagonist, the pyridine derivative MRS 1523 (Li et al., 1998), (−73.8 ± 5.8%, $P < 0.01$ for the transient peak and −82.2 ± 5.2%, $n = 3$ for the sustained phase, respectively). In control experiments, a second Cl-IB-MECA addition in the absence of MRS 1523 induced an increase in I_{sc} and V_t comparable to the first Cl-IB-MECA stimulation (*data not shown*). Together, these data indicate the involvement of A_3 receptors located on the apical membrane of A_6 cells in this process.

The hypothesis that the Cl-IB-MECA dependent I_{sc} peak was due to chloride secretion was supported by experiments in which the response of I_{sc} to Cl-IB-MECA was examined during perfusion of the A_6 cell monolayers with Cl^--free Ringer solution. Substitution of chloride by gluconate reduced both the transient peak and the sustained phase induced by apical Cl-IB-MECA by 67.5 ± 9.1% and 86.6 ± 12.2%, $n = 4$, respectively, demonstrating that the late phase of the I_{sc} response to Cl-IB-MECA reflected a steady-state Cl^- secretion. Moreover, the I_{sc} response to Cl-IB-MECA was also inhibited by

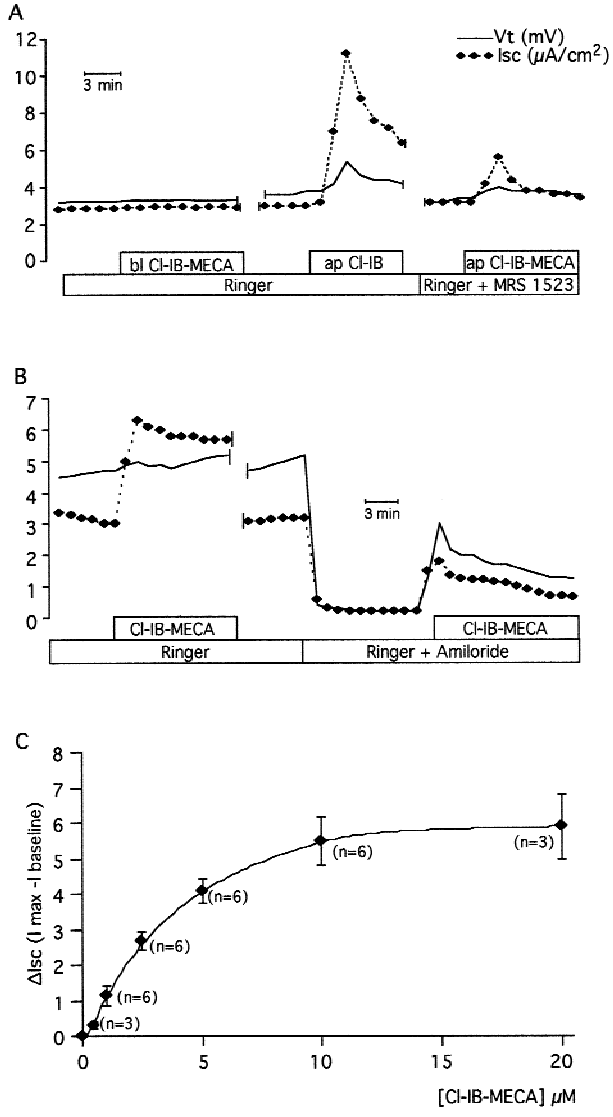

Fig. 1. Effect of Cl-IB-MECA on transepithelial short-circuit current (I_{sc}) and potential difference (Vt) in A_6 monolayers. The preparation was maintained in the open-circuit configuration during the recordings and the transepithelial short-circuit current was measured for 5 sec every 60 sec. (*A*) Representative trace depicting the effect of basolateral or apical Cl-IB-MECA (10 μM) on I_{sc} and Vt in the presence or absence of the selective A_3 antagonist MRS 1523 (10 nM). After an initial period in which I_{sc} was allowed to stabilize, Cl-IB-MECA was added first to the basolateral side and then to the apical side of the monolayer. After reversing the agonist action by washing out Cl-IB-MECA, the selective A_3 antagonist MRS 1523 was added. After 1 hr of preincubation the monolayer was again stimulated with Cl-IB-MECA. Similar results were obtained in three additional experiments. (*B*) Representative trace depicting the response of the amiloride-insensitive component of I_{sc} to 10 μM Cl-IB-MECA added to the apical side of the monolayer. After the first response to I_{sc} to Cl-IB-MECA, 10 μM amiloride was added to the apical compartment 10 min before the readdition of Cl-IB-MECA to the same compartment. (*C*) Dose-response of the peak, transient response of I_{sc} to the following Cl-IB-MECA concentrations (in μM): 0.5, 1, 2.5, 5, 10 and 20. Cl-IB-MECA stimulated the transient response of I_{sc} with an apparent EC_{50} of 3.1 ± 0.2 μM.

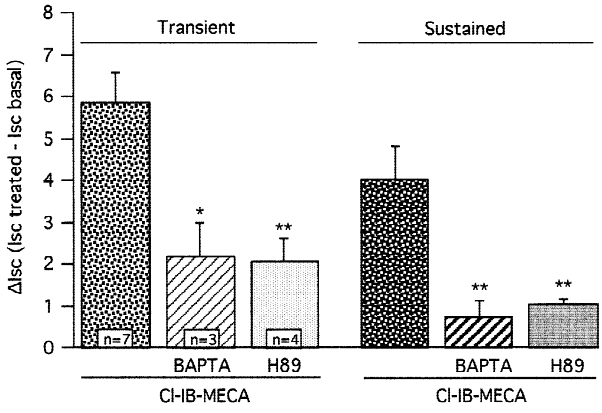

Fig. 2. Effect of inhibition of protein kinase A and calcium increase on Cl-IB-MECA-dependent induction of transepithelial short-circuit current (I_{sc}). A_6 monolayers were first stimulated with 10 μM Cl-IB-MECA and after the I_{sc} and Vt values returned to baseline values the monolayers were preincubated either 15 min with the PKA inhibitor, H89 (1 μM), or 1 hr with the calcium chelator, BAPTA-AM (20 μM), and the response to Cl-IB-MECA was measured again. $^*P < 0.05$, $^{**}P < 0.02$.

the addition to the apical perfusion solution of diphenylamine carboxylic acid (DPC), an inhibitor of some chloride channels (−53.1 ± 10.2% and −43.5 ± 9.5%, of the peak and plateau, respectively, $n = 4$). Further support for this hypothesis was obtained in experiments in which Cl-IB-MECA was still able to induce the I_{sc} increase after apical addition of 10^{-5} M of the Na^+ channel blocker, amiloride (Fig. 1*B*), an experimental procedure used to study the chloride component of I_{sc} (Chalfant et al., 1993 and Verrey, 1994).

The increase of both the transient peak and the sustained phase of I_{sc} were Cl-IB-MECA concentration-dependent: 1 μM led to a mean rise of 2.3 ± 0.3 and 1.4 ± 0.3-fold increase over basal I_{sc} values (calculated at the peak of the transient and after 15 min, respectively, $n = 6$) whereas 10 μM Cl-IB-MECA induced increases of 6.1 ± 0.6 and 3.3 ± 0.4-fold, of the peak and plateau, respectively, $n = 10$. Figure 1*C* shows the dose-response curve of the peak, transient I_{sc} response having a calculated apparent EC_{50} of 3.1 ± 0.2 μM Cl-IB-MECA.

To determine whether the chloride secretion induced by Cl-IB-MECA was mediated by calcium and/or cAMP regulated channels we performed a series of experiments in which the cells were preincubated with either the PKA inhibitor, H-89 (1 μM), or the intracellular calcium chelator, 5,5′-dimethyl BAPTA-AM (20 μM). Both compounds markedly inhibited both the transient and sustained Cl-IB-MECA-dependent increase of I_{sc} (Fig. 2) while not having any significant effect on basal I_{sc}, suggesting that both cell calcium and cAMP play a crucial role in the chain of events that lead to activation of the apical chloride conductance by Cl-IB-MECA.

Intracellular Calcium Measurements

To determine the mechanisms underlying Cl-IB-MECA-dependent alterations in cytosolic calcium levels, calcium was measured microspectrofluorometrically in single cells as outlined in Materials and Methods. One μM Cl-IB-MECA induced a calcium response only when calcium was present in the external medium (1 μM Cl-IB-MECA stimulated $[Ca^{2+}]_i$ from 43.3 ± 7.1 to 196.3 ± 38.2 nM Ca^{2+} when external calcium was 1.4 mM, $n = 10$). This Cl-IB-MECA-dependent induction of $[Ca^{2+}]_i$ was observed in 70% of cells exposed to 1 μM Cl-IB-MECA; the remaining 30% of the cells were unresponsive. This presence of a subset of cells refractory to Cl-IB-MECA may reflect variations in the background level of the endogenous A_3 receptors. Fig. 3*A* shows a typical experiment demonstrating that the $[Ca^{2+}]_i$ response to Cl-IB-MECA increased with increasing external calcium concentration while Fig. 3*B* shows that at a fixed Ringer calcium concentration (1.4 mM) the cells responded to Cl-IB-MECA in a concentration-dependent manner from 0.1 to 10 μM Cl-IB-MECA reaching a plateau between 5 and 10 μM Cl-IB-MECA with a calculated apparent EC_{50} of 1.7 ± 0.4 μM Cl-IB-MECA. Importantly, cells perfused with 1.4 mM external calcium and repeatedly stimulated with the same Cl-IB-MECA concentration (5 min after Cl-IB-MECA removal) always responded with a similar increase in the fluorescence ratio (*data not shown*) demonstrating that there is no reduction in the ability of the cells to respond following repeated Cl-IB-MECA stimulations.

Preincubation with 10 μM $LaCl_3$, a nonspecific inhibitor of Ca^{2+} influx (Pandol et al., 1987), inhibited the calcium response to 5 μM Cl-IB-MECA by 90 ± 5.7% ($P < 0.001$, $n = 3$) while having no effect on resting calcium levels, supporting that Cl-IB-MECA functions by stimulating calcium influx. In contrast, in the same cell preparation the A_1 adenosine agonist, CPA, as we have already reported in the same cell line (Casavola et al., 1996), increased cytoplasmic calcium also in the absence of extracellular calcium, indicating that it raises $[Ca^{2+}]_i$ via the release of calcium from intracellular stores. This response was A_1 receptor specific, as it was inhibited 81.6 ± 7.7% ($P < 0.001$, $n = 5$) by preincubation with 100 nM of the potent A_1 receptor antagonist, CPX (van Galen et al., 1992).

Characterization of the Receptor Type Involved in Cl-IB-MECA Stimulated Calcium Influx

To verify the adenosine receptor subtype mediating the calcium response to Cl-IB-MECA, the effect of preincubation with different antagonists of A_1, A_{2A}/A_{2B} or A_3 adenosine receptors on Cl-IB-MECA stimulated calcium influx was evaluated. The K_i values for these antagonists have been determined in mammalian tissues (*see* Materials and Methods).

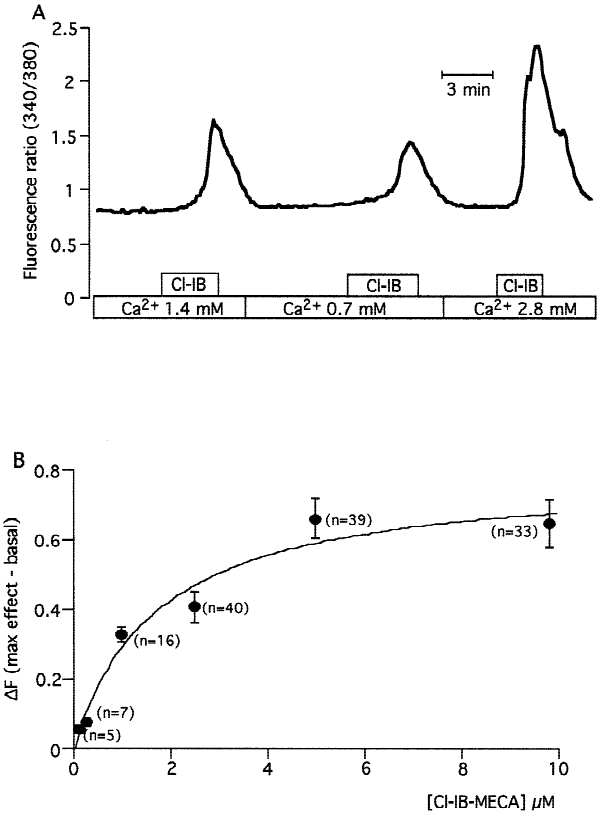

Fig. 3. Effect of Cl-IB-MECA on intracellular calcium concentration ($[Ca^{2+}]_i$). (*A*) Representative trace showing the dependence of the Cl-IB-MECA-induced (5 μM) increase in $[Ca^{2+}]_i$ (fluorescence ratio 340/380) on the calcium concentration present in the Ringer solution perfusing the cells. Cells were continuously perfused with Ringer solutions containing external calcium concentrations as indicated in the figure. (*B*) Effect of increasing concentrations of Cl-IB-MECA on the change in $[Ca^{2+}]_i$ in Ringer with 1.4 mM calcium expressed as the difference (ΔF) of the fluorescence ratio 340/380 at the maximum of the response to Cl-IB-MECA minus the fluorescence ratio observed in baseline conditions. Increasing concentrations of Cl-IB-MECA (0.1, 0.25, 1, 2.5, 5 and 10 μM) stimulated $[Ca^{2+}]_i$ with an apparent EC_{50} of 1.7 ± 0.4 μM.

The effect of the specific A_3 receptor antagonist, MRS 1523, on the Cl-IB-MECA-dependent calcium response was first determined. Figure 4*A* shows the typical effect of a 5 min incubation with 10 nM MRS 1523 on the calcium response induced by 5 μM Cl-IB-MECA. The first peak represents the $[Ca^{2+}]_i$ response of the cell to 5 μM Cl-IB-MECA, which was then removed from perfusion immediately after the peak response. When $[Ca^{2+}]_i$ returned to basal levels, MRS 1523 was added to the perfusate at the indicated time and remained in perfusion for the rest of the experiment. After about 5 min preincubation with the antagonist, the cells were again stimulated with Cl-IB-MECA. In this short incubation MRS 1523 reduced the Cl-IB-MECA response by

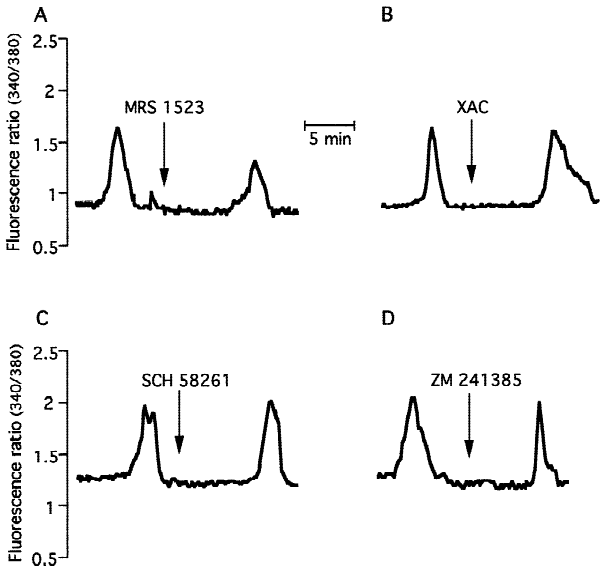

Fig. 4. Effect of various adenosine receptor antagonists on the $[Ca^{2+}]_i$ response induced by Cl-IB-MECA (5 μM). In all plots monolayers were first stimulated with Cl-IB-MECA and changes in $[Ca^{2+}]_i$ measured as described in Fig. 3. Cl-IB-MECA was removed from the perfusate when the transient increase in $[Ca^{2+}]_i$ reached its peak, and each specific adenosine receptor antagonist was added to the perfusate when $[Ca^{2+}]_i$ again returned to basal levels (arrows). The monolayer was perfused with the antagonist for the rest of the experiment. After about 5 min of pretreatment with antagonist the cell was again stimulated with 5 μM Cl-IB-MECA. The antagonists were utilized at the following: the A_3 selective MRS 1523 (10 nM), the moderately A_1 selective xanthine antagonist, XAC (100 nM), the A_{2A} selective antagonists, SCH 58261 (300 nM) and ZM 241385 (100 nM).

−31.6 ± 0.4%, $n = 3$ while a 1 hr preincubation inhibited the response to Cl-IB-MECA by −56.5 ± 17.7%, $n = 3$. This MRS 1523-dependent inhibition was completely reversible in both types of preincubation (*data not shown*). In an additional series of experiments we found that a 5 min preincubation with 2 μM of another selective A_3 antagonist, the 1,4-dihydropyridine derivative MRS 1191 which is 1300-fold selective for human A_3 receptors (Jiang et al., 1996), inhibited the calcium response to 5 μM Cl-IB-MECA by 36.8 ± 7.7% ($n = 4$, $P < 0.05$).

In analogous experiments (Fig. 4*B*) it can be seen that treatment with 100 nM of the moderately A_1 selective xanthine antagonist, XAC (van Galen et al., 1992), did not significantly affect the calcium response induced by 5 μM Cl-IB-MECA (+2.3 ± 8.7%, $n = 6$, NS). Another A_1 selective antagonist, CPX, also had no effect on the Cl-IB-MECA induced calcium effect (−10.4 ± 8.5%, $n = 6$, NS). Moreover, neither 300 nM SCH 58261 (Fig. 4*C*), a specific A_{2A} adenosine receptor antagonist (Zocchi et al., 1996), nor 100 nM ZM 241385 (Fig. 4*D*), an antagonist of both A_{2A} and A_{2B} receptors (Poucher et al., 1995; Ji & Jacobson, 1999), significantly altered the calcium response to 5 μM Cl-IB-MECA (+18.4 ± 19.7%, NS, $n = 6$ and −11.4 ± 11.7%, NS, $n = 7$, for SCH 58261 and ZM 241385, respectively). ZM 241385 at a 20-fold higher concentration still had no effect on the Cl-IB-MECA-dependent calcium response (+3.3 ± 8.8%, NS, $n = 4$). Additionally, A_2 receptor activation with either the nonselective agonist, NECA (used from 1 to 10 μM), or the A_{2A} selective agonist, DPMA (10 μM) (Daly & Jacobson, 1995), did not induce a change in intracellular calcium in cells that were responsive to 5 μM Cl-IB-MECA. Importantly, as can be seen in Fig. 4, pretreatment with these various antagonists had no effect on basal $[Ca^{2+}]_i$.

Signal Transduction Systems Involved in Calcium Responses to Cl-IB-MECA

We then tried to elucidate the signal transduction mechanisms involved in the Cl-IB-MECA-dependent stimulation of $[Ca^{2+}]_i$. To date, published data have suggested that coupling of both A_1 and A_3 receptors to G_i/G_o protein in many cellular systems stimulates inositol 1,4,-trisphosphate production (for review *see:* Palmer & Stiles, 1995). To explore this possibility in A_6 cells, pertussis toxin (PTX) pretreatment (200 ng/ml overnight) was used to prevent activation of G_i and/or G_o during cell stimulation. PTX functionally uncouples these proteins from cell surface receptors by ADP-ribosylating the α-subunit of G_i/G_o (Ui et al., 1984). While the 5 μM Cl-IB-MECA-dependent calcium response was only partially and no significantly inhibited by PTX pretreatment in A_6 cells, the inhibitory effect of PTX on the calcium response induced by 1 μM of the A_1 adenosine agonist CPA, was almost complete (from a Cl-IB-MECA-dependent ΔF of 0.47 ± 0.07 to 0.38 ± 0.06 before and after PTX treatment, respectively, $n = 5$, NS *vs.* a CPA-dependent ΔF of 0.34 ± 0.08 to 0.03 ± 0.01 before and after PTX treatment, respectively, $n = 3$, $P < 0.001$).

These findings suggest that in A_6 cells Cl-IB-MECA, differently from CPA, acts mainly via a G_i-protein insensitive signal transduction pathway. To test the possibility that the calcium responses to Cl-IB-MECA could be modulated by cAMP/PKA activation, the calcium response to Cl-IB-MECA was analyzed in the absence or presence of 10 μM forskolin, an agent that stimulates the production of cAMP, 10 nM rolipram, an agent that inhibits cAMP-specific phosphodiesterases, or 1 μM of the inhibitor of PKA, H-89 (Fig. 5). Preincubation with H-89 inhibited the calcium response to Cl-IB-MECA by 45% while having no effect on basal calcium levels. Preincubation with forskolin elicited a transient calcium response ($\Delta F = 0.61 \pm 0.13$) in five of thirteen cells examined. In the eight cells in which forskolin had no effect on the basal levels of calcium, it significantly potentiated the effect of 5 μM Cl-IB-MECA by 42.22 ± 6.44%, $n = 8$ (Fig. 5). Preincubation with

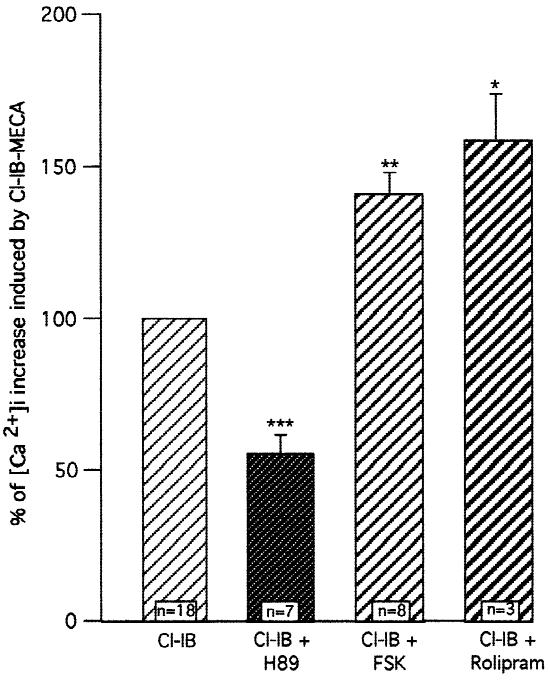

Fig. 5. Effect of H89 (1 μM), Forskolin (10 μM) or Rolipram (10 nM) on the $[Ca^{2+}]_i$ response induced by 5 μM Cl-IB-MECA. The substances were added 10 min before the addition of Cl-IB-MECA. $*P < 0.02$, $**P < 0.01$, $***P < 0.001$.

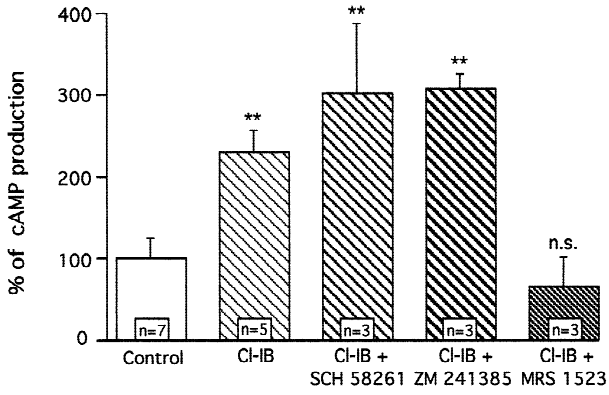

Fig. 6. Effect of 5 μM Cl-IB-MECA and various adenosine receptor antagonists on intracellular cAMP generation in A_6 cell monolayers. Cl-IB-MECA was added to the apical side of the monolayers for 15 min before the samples were analyzed for cAMP content as described in Materials and Methods. When present, the antagonists were added 15 min before the addition of Cl-IB-MECA at the same concentrations as in Fig. 4. Preincubation with all of the antagonists was without effect on the basal cAMP levels. $**P < 0.01$.

rolipram had no effect on the basal levels of calcium in any cells. Interestingly, preincubation with rolipram converted Cl-IB-MECA nonresponding cells into responding cells whereas in cells already responsive to 5 μM Cl-IB-MECA rolipram treatment increased their calcium response (Fig. 5). A 3 hr preincubation with 100 ng/ml cholera toxin, which potentiates hormone-stimulated elevation of cellular cAMP by catalyzing ADP-ribosylation of G_s, significantly increased the calcium response to Cl-IB-MECA (+33.6 ± 8.1%, $P < 0.02$, $n = 4$), indicating an involvement of a cholera toxin-sensitive G-protein in Cl-IB-MECA-induced Ca^{2+} influx in A_6 cells. Altogether, these data support the hypothesis that the Cl-IB-MECA-dependent increase in $[Ca^{2+}]_i$ occurs mainly through the activation of adenylate cyclase/PKA.

To provide additional support for this hypothesis, we determined the effect of Cl-IB-MECA on the levels of intracellular cAMP. As shown in Fig. 6, addition of 5 μM Cl-IB-MECA to the apical side of the monolayer resulted in a significative increase of cAMP that was prevented by the A_3 antagonist, MRS 1523 (10 nM). Neither the selective A_{2A} antagonist, SCH 58261 (300 nM), nor the mixed A_{2A}/A_{2B} antagonist, ZM 241385 (100 nM), altered this response. Pretreatment of the monolayer with these antagonists had no effect on basal cAMP levels (*data not shown*).

Finally, to investigate the potential interaction of A_3 adenosine receptors with receptors that act through activation of adenylate cyclase (stimulation of G_s), we measured the modulation by Cl-IB-MECA of arginine vasopressin (AVP)-dependent cAMP accumulation. AVP is known to increase cAMP levels in A_6 cells (Casavola et al. 1992) by stimulation of adenylate cyclase via coupling to G_s. The effect of AVP (0.5 μM) alone or in combination with Cl-IB-MECA on intracellular cAMP concentration are shown in Fig. 7. Treatment of A_6 cells with AVP alone significantly increased cAMP levels. This AVP-induced stimulation of cAMP was potentiated additively by Cl-IB-MECA suggesting that the A_3 receptor acts mainly through a G_s/PKA mechanism in these cells. Importantly, 5 μM CPA, a A_1 adenosine agonist known to act via a G_i-dependent mechanism had no effect on basal cAMP levels (0.17 ± 0.04 *vs.* 0.12 ± 0.03 pmol/filter/10 min for basal and CPA stimulated cells, respectively, $n = 3$, NS) while it completely inhibited the AVP-dependent induction of cAMP levels (0.36 ± 0.15, *vs.* 0.16 ± 0.05 pmol/filter/10 min for AVP and AVP + CPA stimulated cells, respectively, $n = 3$, $P < 0.01$). Altogether, these data confirm that in A_6 cells the A_1 receptor acts primarily through a G_i-dependent mechanism and demonstrate that the A_3 receptor does not have a G_i-dependent component for its action.

Discussion

The expression of the A_3 receptors in the kidney has been demonstrated by Zhou et al. (1992). While functional studies have been conducted in cells that have been transfected with the A_3 receptor (Linden et al., 1993; Salvatore et al., 1993), there is limited information re-

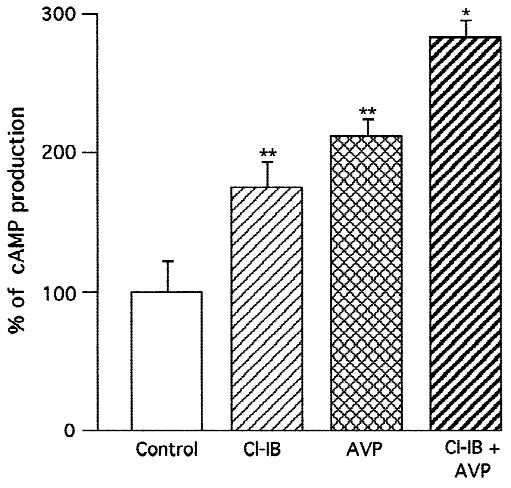

Fig. 7. Effect of Cl-IB-MECA on vasopressin (AVP)-stimulation of cAMP accumulation. AVP (0.5 μM) was added to the monolayers alone or in combination with 5 μM Cl-IB-MECA for 15 min before the samples were analyzed for cAMP content. Each bar is the mean ± SE of at least three determinations. Both Cl-IB-MECA and AVP significantly increased cAMP compared to control values (**$P < 0.01$). Cl-IB-MECA significantly stimulated the AVP-dependent cAMP increase (*$P < 0.02$).

garding the functional characteristics of native A_3 receptors in renal cell lines.

We have previously demonstrated that A_6 cells, a cell line derived from the kidney of *Xenopus laevis* that is commonly used as a model of the mammalian collecting duct, contain both A_1 and A_2 receptors (Casavola et al., 1996). The A_1 receptors, located on the apical membrane, act via mobilization of intracellular calcium whereas A_2 receptors are located on the basolateral surface and stimulate cAMP production and, secondarily, transepithelial sodium transport (Casavola et al., 1997). In the present study the use of Cl-IB-MECA, an agonist that is highly selective for the A_3 receptor (Jacobson et al., 1995), and A_3 selective antagonists such as MRS 1523 (Li et al., 1998) and MRS 1191 (Jiang et al., 1996) has permitted us to investigate the potential role and mechanism of action of the A_3 receptor in the kidney.

Chloride Secretion Activated by Cl-IB-MECA

In the present report we show that only apical addition of Cl-IB-MECA results in an increase in the I_{sc} that was significantly inhibited by MRS 1523. That this Cl-IB-MECA-dependent increase in I_{sc} is due to a chloride secretion is supported by (i) the application of amiloride to the apical side of the monolayer had no significant effect on the Cl-IB-MECA-induced I_{sc} and (ii) the Cl-IB-MECA-induced I_{sc} was significantly reduced both in chloride-free media and after treatment with DPC, a blocker of Cl^- channels in numerous Cl^--transporting epithelia (Di Stefano et al., 1985). Activation of Cl^- secretion by different agonists has been described in A_6 cells: chloride is transported by two process including a $Na^+/K^+/2Cl^-$ cotransporter in the basolateral membrane (Yanase & Handler, 1986) and two different Cl^- channels at the apical membrane regulated either by calcium or cAMP or both (Chalfant et al., 1993; Verrey, 1994; Nisato & Marunaka, 1997; Atia, Zeiske & Van Driessche, 1999).

The recent report that Cl-IB-MECA induces chloride secretion in nonpigmentated ciliary epithelial cells (Mitchell et al., 1999) provides additional evidence that A_3 receptor stimulation by an autocrine/paracrine mechanism may regulate cell volume and/or liquid secretion.

The chloride secretion induced by Cl-IB-MECA in A_6 cells was markedly inhibited by pretreatment with either BAPTA or by H-89 suggesting that both cell calcium and cAMP play a crucial role in the chain of events leading to activation of the apical Cl-IB-MECA-induced chloride secretion. These data, together with the evidence discussed later demonstrating a potentiating effect of cAMP on calcium influx, suggest that PKA activity may be required for elevation of the cytosolic calcium level and subsequent activation of calcium-activated chloride channels.

Calcium Entrance Activated by Cl-IB-MECA

Another important finding of the present study is the demonstration that activation by Cl-IB-MECA induces an A_3 receptor-dependent calcium entry. The supporting evidence includes (i) Cl-IB-MECA induced a calcium response only when calcium was present in the external medium, and this increase was almost completely inhibited by $LaCl_3$, an agent known to block all Ca^{2+} influx mechanisms across the plasma membrane (Pandol et al., 1987); (ii) the calcium influx was dependent on the external calcium concentration; (iii) both of the A_3 selective antagonists, MRS 1523 and MRS 1191, significantly inhibited the Cl-IB-MECA induced calcium response; (iv) neither A_{2A} nor A_{2B} antagonists had any effect on this calcium response.

While both the A_3 adenosine agonist, Cl-IB-MECA, and the A_1 agonist, CPA, induce an increase in $[Ca^{2+}]_i$, the mechanism underlying the calcium response to activation of each adenosine receptor subtype and the signal transduction mechanism regulating the receptor-specific increase in calcium were different. Contrary to Cl-IB-MECA, in A_6 cells the adenosine A_1 specific agonist, CPA, increases $[Ca^{2+}]_i$ in absence of external medium calcium (Casavola et al. 1996). Whereas the calcium response to Cl-IB-MECA was only partially (~20%) and not significantly inhibited by PTX, the CPA-induced calcium response was almost completely (~90%) inhibited by PTX as has been reported in other cell systems

(Palmer & Stiles, 1995). Pharmacological stimulation of intracellular cAMP by forskolin or by cholera toxin treatment significantly stimulated the calcium response to Cl-IB-MECA while H-89, a known inhibitor of PKA, inhibited the Cl-IB-MECA specific response. Forskolin treatment inhibits CPA-dependent calcium response by 46.4 ± 1.9%, $n = 3$ ($P < 0.001$). These data strongly suggest that in A_6 cells A_3 receptor activation acts through a G_s/PKA pathway while the A_1 receptor acts through activation of the G_i pathway. This conclusion is supported by the data showing that Cl-IB-MECA incubation is additive to AVP-dependent cAMP production (Fig. 7) while CPA inhibited the cAMP production induced by AVP.

That Cl-IB-MECA acts by an apical membrane A_3 receptor through a G_s/PKA pathway is further supported by the findings that only apically addition of Cl-IB-MECA to the A_6 cell monolayers significantly increased cellular cAMP content. This increase was almost completely abrogated by the A_3 antagonist, MRS 1523, while neither the selective A_{2A} antagonist, SCH58261, nor the antagonist of both A_{2A} and A_{2B} receptors, ZM241385, had any effect (*see* Fig. 6).

Increases in intracellular calcium have been demonstrated to modulate the cAMP cascade (Tang, Krupinski & Gilman, 1991). In our model system, we can exclude the possibility that adenylate cyclase is secondary to the increase of calcium levels since the pretreatment with rolipram, an inhibitor of phosphodiesterase, not only increased the calcium response to Cl-IB-MECA but also converted Cl-IB-MECA nonresponding cells into responding cells. Altogether these results support the hypothesis that the Cl-IB-MECA-dependent $[Ca^{2+}]_i$ increase is positively modulated through the activation of adenylate cyclase/PKA in A_6 cells. The G_s pathway could be the branch linking the A_3 receptors to both adenylate cyclase (AC) and calcium channels, since upregulation of AC has been demonstrated to increase the phosphorylation of voltage-dependent L-type calcium channels (Yatani & Brown, 1989). Further, in rat and rabbit cortical collecting ducts a permissive role of cAMP in $[Ca^{2+}]$ increase in presence of external calcium has been demonstrated (Breyer, 1991; Siga, Champigneulle & Imbert-Teboul, 1994) and in A_6 cells Nisato and Marunaka (1997) have proposed a permissive role of cAMP/PKA in opening calcium channels to provide calcium entry even in the absence of the Ca^{2+} mobilizing, IP_3 pathway.

While the results regarding the signal transduction mechanism in A_6 cells induced by the A_1 agonist, CPA, are in accordance with data obtained in other cellular systems (reviewed in Olah & Stiles, 1992; Palmer & Stiles, 1995) our results regarding the predominant involvement of PKA-regulated calcium influx induced by Cl-IB-MECA in A_6 cells differ from those obtained in several other cellular systems in which calcium release from internal stores mediated by a PLC pathway is the main mechanism of Cl-IB-MECA action (Ramkumar et al., 1993; Abbracchio et al., 1995). One explanation for the different mechanism of action of Cl-IB-MECA in A_6 cells could be due essentially to the diversity of the A_3 receptor in amphibian cells that to date has not been characterized either pharmacologically or physiologically. Importantly, in guinea pig hippocampal pyramidal neurons Fleming and Mogul (1997) reported a mechanism similar to that reported here: that adenosine A_3 receptors stimulate a calcium current that is prevented by inhibiting PKA. These data, together with ours, suggest a plasticity in A_3 post-receptor signaling mechanisms that could be tissue-dependent. This certainly is an important question worthy of more detailed investigation in the future.

The different signaling pathway induced by Cl-IB-MECA in these cells could be a result of the presence of both A_1 and A_3 receptors on the same cell surface of the cell. The co-existence in the same cell of two distinct mechanisms for increasing $[Ca^{2+}]_i$ following the activation of the A_1 and A_3 receptors opens the question of their physiological importance. The complexity in post-receptor transduction mechanisms activated by two different receptors could permit a wider variety of cross-talk with and control of other hormone actions with respect to the control of kidney function. To further evaluate the possible interaction of different receptors and their post-receptor mechanisms we analyzed the interaction of the A_1 and A_3 receptors with vasopressin (AVP) receptor activation. The data concerning the interaction of the A_1 and A_3 receptors with AVP receptor activation is an example of the different modulating role that the two adenosine receptor can effect on renal function via cross-talk. Having two receptors for the same hormone in the same cell with such different signaling transduction pathways permits the detailed, finely controlled pleiotrophic responses necessary in natural situations. Vasopressin is known to stimulate hydraulic water transport and electrogenic sodium transport in the collecting duct epithelial cells and the findings that Cl-IB-MECA potentiate the cAMP production of AVP, while the A_1 agonist, CPA, had an opposite effect, are an important example of the modulatory role of the adenosine receptors of kidney cell function.

In conclusion, the data reported here suggest that the Cl-IB-MECA-induced calcium increase is mediated mainly by a G_s/PKA mechanism. We can hypothesize that the predominant mechanism induced by Cl-IB-MECA involves a calcium entry. On the basis of our physiological data, we cannot, however, rule out the possibility that small amounts of intracellular calcium can be redistributed locally to trigger the predominant calcium entry.

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