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Adenosine stimulates Ca^{2+} fluxes and increases cytosolic free Ca^{2+} in cultured rat mesangial cells

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Adenosine has been associated with cellular Ca^{2+} metabolism in some cell types. Since adenosine is able to contract glomerular mesangial cells in culture, and since Ca^{2+} is the main messenger mediating contractile responses, we studied the effect of adenosine on $^{45}Ca^{2+}$ movements into and out of mesengial cells and on the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Adenosine at 0.1 mM increased $^{45}Ca^{2+}$ uptake (basal, 9993 ± 216; + adenosine, 14823 ± 410 d.p.m./mg; $P < 0.01$) through verapamil-sensitive Ca^{2+} channels. These channels seem to be of the A_1-adenosine receptor subtype. Adenosine also stimulated $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded mesangial cells. This effect was accompanied by a net depletion of intracellular $^{45}Ca^{2+}$ content under isotopic equilibrium conditions (basal, 24213 ± 978; + adenosine, 18622 ± 885 d.p.m./mg; $P < 0.05$). The increase in $^{45}Ca^{2+}$ efflux was inhibited by a Ca^{2+}-free medium or in the presence of 10 μM-verapmil. However, the intracellular Ca^{2+}-release blocker TMB-8 (10 μM) only partially inhibited the adenosine-stimulated $^{45}Ca^{2+}$ efflux. In addition, adenosine induced an elevation in $[Ca^{2+}]_i$ in mesangial cells with an initial transient peak within 15 s (basal, 113 ± 7; adenosine, 345 ± 46 nM), and a secondary increase which was slower (3–4 min) and of lower magnitude than the initial peak (250 ± 21 nM). In summary, adenosine elevates $[Ca^{2+}]_i$ and stimulates both Ca^{2+} uptake from the extracellular pool and Ca^{2+} efflux from intracellular pools in mesangial cells. The Ca^{2+} release from internal stores is produced by a combination of a TMB-8-inhibitable and a non-TMB-8-inhibitable mechanism, and seems to be dependent on Ca^{2+} influx.

INTRODUCTION

Most cells have specific surface membrane receptors for adenosine and adenosine analogues which are coupled to the adenylate cyclase membrane system [1,2]. Although adenosine and adenosine derivatives are able to modify cyclic AMP levels in a wide variety of cell types [1,3], the actual relevance of cyclic AMP as a second messenger mediating the physiological responses to adenosine has not been demonstrated in all tissues or cell systems [4–7].

However, there is increasing evidence for the coupling of adenosine receptors to ion channels [8–10] and for adenosine-induced changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in some cell types [4,11–13]. In the kidney, Ca^{2+} appears to play an important role in the effects of adenosine. One of the major renal effects observed after adenosine administration is a transient renal vasoconstriction. The vasoconstriction induced by adenosine in the kidney seems to be dependent on voltage-operated Ca^{2+} channels, since it is inhibited by verapamil [14,15]. We have previously demonstrated that adenosine also induces glomerular and mesangial cell contraction, and this action seems to depend on extracellular Ca^{2+} [16]. Intraglomerular mesangial cells, which are important functional modulators of the glomerular filtration rate, show interesting contractile properties that resemble those of smooth muscle cells. Mesangial cells are able to contract in response to vasoactive agonists such as angiotensin II, [Arg^8]vasopressin (AVP), endothelin, platelet-activating factor and others, and a common feature in the action of these agents in mesangial cells is Ca^{2+} mobilization accompanied by changes in $[Ca^{2+}]_i$ [17,18]. This Ca^{2+} mobilization has been reported for angiotensin II and endothelin to be dependent on phospholipase C activation and subsequent hydrolysis of PtdInsP_2 [19].

To evaluate the role of Ca^{2+} in the mesangial cell response to adenosine, we studied Ca^{2+} movements across the plasma membrane by measuring Ca^{2+} influx and efflux using $^{45}Ca^{2+}$ as a radioactive indicator. We also measured changes in $[Ca^{2+}]_i$ induced by adenosine in mesangial cells using the fluorescent dye Fura-2.

EXPERIMENTAL

Materials

Adenosine, PIA (N^6-R-1-methyl-2-phenylethyladenosine), NECA (adenosine-5-N-ethylcarboxamide), TMB-8 [3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester hydrochloride], ionophore A23187, AVP, collagenase type IA (from *Clostridium histolyticum*), EGTA and L-glutamine were purchased from Sigma (St. Louis, MO, U.S.A.). Verapamil was provided by Knoll–Made, Madrid, Spain. Penicillin was obtained from Laboratories Level SA, Barcelona, Spain. Streptomycin sulphate was obtained from Antibioticos SA, Madrid, Spain. RPMI 1640 medium, Hanks balanced salt solution and fetal calf serum were obtained from Flow Laboratories, Woodcock Hill, Herts., U.K. Fura-2/AM was purchased from Molecular Probes (Junction City, OR, U.S.A.), and $^{45}CaCl_2$ was from Amersham. PD116,948 (8-cyclopenthyl-1,3-dipropylxanthine) was a gift from Warner–Lambert, Parke Davis, Ann Arbor, MI, U.S.A.

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; AVP, [Arg^8]vasopressin; PIA, N^6-R-I-methyl-2-phenylethyladenosine; NECA, adenosine-5-N-ethylcarboxamide; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester hydrochloride.

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Mesangial cell culture

Glomeruli were isolated from kidneys of Wistar rats by successive mechanical sieving (150 and 50 μm). Isolated glomeruli were treated with collagenase (300 units/ml), plated in 35 mm plastic tissue culture dishes (Costar) and maintained in the conditions previously described [17,20]. The culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (1 mM), penicillin (0.66 μg/ml) and streptomycin sulphate (60 μg/ml), and buffered with 20 mM-Hepes, pH 7.2. The culture medium was changed every 2 days. Studies were performed on day 21 or 22, by which time epithelial cells were no longer detected in the culture dishes. The identity of the cells was confirmed by morphological and functional criteria as described previously [17,20].

$^{45}Ca^{2+}$ uptake

After washing the cells twice with Tris/glucose buffer (Tris 20 mM, NaCl 130 mM, KCl 10 mM, sodium acetate 10 mM and glucose 5 mM, pH 7.4), cells were preincubated in the same buffer for 20 min. $^{45}Ca^{2+}$ uptake experiments were performed by incubating the cells in Tris/glucose buffer with 2.5 mM-$CaCl_2$ containing 4 μCi of $^{45}Ca^{2+}Cl_2$/ml in the presence or the absence of adenosine or KCl for 30 s at 37 °C. The uptake was interrupted by aspirating the medium containing $^{45}Ca^{2+}$ and by sequentially dipping the dish once in each of two beakers containing 25 ml of ice-cold buffer without Ca^{2+} and with 2.5 mM-EGTA. Cells were solubilized in 0.1% SDS and samples were taken for scintillation counting and protein determination. All experiments were done in triplicate or quadruplicate.

$^{45}Ca^{2+}$ efflux

Mesangial cells were loaded with 4 μCi of $^{45}Ca^{2+}Cl_2$/ml by incubating the cultures overight in the presence of the isotope. The $^{45}Ca^{2+}$ efflux experiment was carried out at 37 °C in a Tris/glucose buffer containing 2.5 mM-$CaCl_2$ and the indicated agonists after removing the labelling medium and rapidly washing the cultures with 12 × 2 ml of the same buffer. The whole washing procedure took exactly 2 min. At the times indicated, samples of 0.2 ml were taken for scintillation counting and were replaced by 0.2 ml of fresh medium.

$[^{45}Ca^{2+}]_i$ under isotopic equilibrium conditions

Mesangial cells were loaded overnight with 4 μCi of $^{45}Ca^{2+}$ Cl_2/ml. Loaded cells were then incubated with the various agents or the corresponding solvents in the presence of the isotope. After 5 min of incubation at 37 °C, cultures were washed as described in the $^{45}Ca^{2+}$ uptake experiments. The radioactivity remaining in the cells and cellular proteins were determined as described above. All experiments involved triplicate or quadruplicate determinations.

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

In order to measure $[Ca^{2+}]_i$, cell monolayers were grown on plastic coverslips. After washing with Tris/glucose buffer containing 2.5 mM-$CaCl_2$, pH 7.4, the coverslips were incubated for 45 min at 37 °C in the same buffer containing 4 μM-fura-2/AM. The monolayers were then washed three times with the same buffer containing no fura-2 and incubated for another 15 min at 37 °C to allow the non-hydrolysed fura-2 to diffuse from the cells. Before measurement, the coverslips were rinsed again with Tris/glucose buffer containing 2.5 mM-Ca^{2+}. Fluorescence was measured at 37 °C using a fluorescence spectrophotometer equipped with a thermostastically controlled cuvette holder (Perkin–Elmer LS-5) at an emission wavelength of 500 nm. Excitation wavelengths of 342 and 380 nm were chosen to monitor the Ca^{2+}-induced shift in fura-2 fluorescence as determined by previous calibration determinations. Autofluorescence was measured in similar cells which had not been loaded with fura-2, and was always below 10% of the total fluorescence of fura-2-loaded cells. $[Ca^{2+}]_i$ was calculated using a K_D of 224 nM, as described by Grynkiewicz *et al.* [21]. Under the conditions used in the present experiments, the distribution of fura-2 was homogeneous in more than 95% of the cells.

Statistics

Data are shown as means ± S.E.M. Statistical differences between means were assessed by either the Kruskal–Wallis test or one-way/two-way analyses of variance followed by a multiple means comparisons test (Scheffé's test). A P value < 0.05 was considered as statistically significant.

RESULTS

Effect of adenosine and KCl on $^{45}Ca^{2+}$ uptake into mesangial cells

Adenosine induced a dose-dependent increase in $^{45}Ca^{2+}$ uptake into mesangial cells after 30 s of incubation, and this was significant at concentrations higher than 1 μM (Table 1). Adenosine at 0.1 mM increased $^{45}Ca^{2+}$ uptake by 40% over basal values, and a similar increment was obtained when cells were depolarized by 60 mM-KCl. Both adenosine- and KCl-stimulated $^{45}Ca^{2+}$ uptake were completely blocked by the voltage-operated Ca^{2+} channel inhibitor verapamil (10 μM). Treatment with verapamil alone induced a slight but not significant decrease in $^{45}Ca^{2+}$ uptake in the control cultures (Table 2). The adenosine analogue PIA, a relatively specific A_1-adenosine-receptor agonist, also increased $^{45}Ca^{2+}$ uptake by mesangial cells, whereas this increase was not observed when the cells were treated with NECA, a relatively selective A_2-adenosine-receptor agonist (Table 3). The adenosine-induced $^{45}Ca^{2+}$ uptake increase was completely blocked by PD116,948, a selective antagonist of the A_1-subtype receptor (Table 3).

Effect of adenosine and KCl on $^{45}Ca^{2+}$ efflux from mesangial cells

The efflux of $^{45}Ca^{2+}$, monitored as release of radioactivity into the medium at different times of incubation, showed a pattern similar to that previously reported for other cells [22]. Ionophore A23187 (10 μM) [23] and AVP (0.1 μM) were used as positive controls, and both induced a significant increase in $^{45}Ca^{2+}$ release

Table 1. Effect of adenosine on Ca^{2+} uptake by mesangial cells

Mesangial cells were incubated for 30 s with different concentrations of adenosine. The numbers of experiments are shown in parentheses; each experiment was done in triplicate or quadruplicate. Significant differences are shown [*P < 0.05, one-way analysis of variance and multiple means comparison test (Scheffé test)] compared with Ca^{2+} uptake.

[Adenosine] (μM)	$^{45}Ca^{2+}$ uptake (d.p.m./mg of protein)
0	8416 ± 225 (6)
0.1	8847 ± 320 (5)
1.0	8729 ± 216 (5)
10	9950 ± 411* (6)
100	11196 ± 286* (6)

Table 2. Effect of adenosine and KCl, in the presence or the absence of verapamil, on Ca^{2+}, uptake by mesangial cells

Mesangial cells were incubated for 30 s with adenosine or KCl. In some experiments, cells were preincubated for 10 min with the Ca^{2+}-channel blocker verapamil (10 μM) in Tris/glucose buffer. The number of experiments is shown in parenthesis; each experiment was done in triplicate or quadruplicate. Significant differences are indicated [$*P < 0.05$, one way analysis of variance and multiple means comparison test (Scheffé test)] compared with Ca^{2+} uptake. Significant differences from cells in the absence of verapamil are also indicated († $P < 0.05$).

	$^{45}Ca^{2+}$ uptake (d.p.m./mg of protein)	
	−Verapamil	+Verapamil
Basal	9993±216 (4)	8507±302 (3)
KCl (60 mM)	13120±816* (4)	9100±108† (3)
Adenosine (0.1 mM)	14823±410* (4)	8516±296† (3)

Table 3. Effects of adenosine, adenosine analaogues and an adenosine antagonist on Ca^{2+} uptake by mesangial cells

Mesangial cells were incubated for 30 s with adenosine or the adenosine analogues PIA or NECA. In some experiments with adenosine cells were preincubated for 10 min with the selective antagonist of the A_1-subtype receptor, PD116,948 (10 nM). The number of experiments is shown in parentheses; each experiment was done in triplicate or quadruplicate. Significant differences are indicated [$P < 0.05$, one-way analysis of variance and multiple means comparison test (Scheffé test)] compared with basal Ca^{2+} uptake.

	$^{45}Ca^{2+}$ uptake (d.p.m./mg of protein)
Basal	9903±216 (4)
PIA (1 μM)	11140±310* (5)
NECA (1 μm)	9009±268 (4)
Adenosine (0.1 mM)	14320±316* (4)
Adenosine+PD116,948	8754±226 (4)

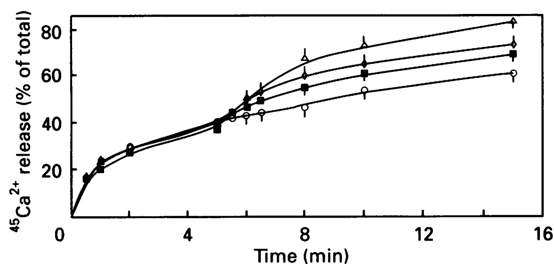

Fig. 1. $^{45}Ca^{2+}$ release from $^{45}Ca^{2+}$-loaded mesangial cells at different times of incubation

After washing the cells, the $^{45}Ca^{2+}$ efflux study was started by addition of fresh medium, and the agonists 0.1 mM-adenosine (◇, $n = 10$), 0.1 μM-AVP (△, $n = 7$) or 60 mM-KCl (⊠, $n = 9$), or the solvent (○, $n = 6$), was added after 5 min of incubation in this medium. Data are means±S.E.M. Each curve was significantly different ($P < 0.05$, two-way analysis of variance) from the other curves.

from the cells (results not shown). Adenosine (0.1 mM) caused a significant increase in $^{45}Ca^{2+}$ efflux from mesangial cells, although the stimulation was of less magnitude than that elicited by the

Table 4. Effect of pretreatment with verapamil or TMB-8 on Ca^{2+} release stimulated by adenosine or KCl

After washing the cells, the $^{45}Ca^{2+}$ efflux study was started by addition of fresh medium, and the agonists [0.1 mM-adenosine (ADO), 0.1 μM-AVP) or 60 mM-KCl] were added after 5 min of the incubation in this medium. The results represent the accumulation in the incubation medium of $^{45}Ca^{2+}$ released from $^{45}Ca^{2+}$-loaded mesangial cells 5 min after the addition of 60 mM-KCl or 0.1 mM-adenosine. Cells were pretreated for 10 min with 10 μM-verapamil or 10 μM-TMB-8. Data are means±S.E.M. of four experiments per group, each done in triplicate or quadruplicate. Significant differences ($*P < 0.05$) versus control values, and significant differences (†$P < 0.05$) versus values without TMB-8 or verapamil [one-way analysis of variance and multiple means comparison test (Scheffé test)] are shown.

	$^{45}Ca^{2+}$ release (d.p.m./mg of protein)		
Agonist	Control	+TMB-8	+Verapamil
	54320±560	55010±261	52510±509
ADO	64750±366*	59406±210*†	55210±339†
KCl	60306±256*	60506±305*	53727±216*
AVP	72150±342*	61210±360*†	68972±342*†

Ca^{2+}-releasing hormone AVP. To discard the possibility of an increase in $^{45}Ca^{2+}$ release from extracellularly bound $^{45}Ca^{2+}$, 0.1 mM-adenosine was added 5 min after the onset of the $^{45}Ca^{2+}$ efflux, a time by which the 'very fast' surface $^{45}Ca^{2+}$ pool has been almost totally mobilized [22]. As shown in Fig. 1, when added after 5 min from the beginning of the experiment, 0.1 mM-adenosine induced a rapid increase in the rate of $^{45}Ca^{2+}$ efflux. However, the rate of $^{45}Ca^{2+}$ efflux induced by adenosine was lower than that introduced by AVP (Fig. 1).

Adenosine failed to stimulate $^{45}Ca^{2+}$ release from the cells when a Ca^{2+}-free medium was used for the experiment (results not shown). Since $^{45}Ca^{2+}$ efflux induced by adenosine seemed to be dependent on extracellular Ca^{2+}, we decided to assess whether a mere stimulation of Ca^{2+} uptake could account for an increase in Ca^{2+} release. Thus $^{45}Ca^{2+}$ efflux experiments were performed in the presence of 60 mM-KCl, which depolarizes plasma membranes [24] and, as shown in Table 2, induces in mesangial cells similar effects on $^{45}Ca^{2+}$ uptake as adenosine. KCl also stimulated $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded mesangial cells, although the accumulation of the isotope in the medium induced by KCl was less than that induced by adenosine (Fig. 1). Further analysis of this response was obtained when the Ca^{2+}-channel antagonist verapamil (10 μM) and the relatively specific intracellular Ca^{2+}-release blocker TMB-8 (10 μM) were used in these experiments. Table 4 shows $^{45}Ca^{2+}$-release from $^{45}Ca^{2+}$-loaded mesangial cells and its accumulation in the incubation medium after 5 min in the presence of adenosine or KCl, following preincubation with or without verapamil or TMB-8. Adenosine-induced stimulation of $^{45}Ca^{2+}$ release was inhibited completely by verapamil but only partially by TMB-8. KCl-induced stimulation of $^{45}Ca^{2+}$ release from mesangial cells was inhibited by verapamil but not by TMB-8. AVP, a hormone that releases Ca^{2+} from intracellular stores, induced a Ca^{2+} release of greater magnitude than that induced by adenosine or KCl. This Ca^{2+} release was markedly but not completely blocked by TMB-8, and only slightly blocked by verapamil (Table 4).

Effects of adenosine on $^{45}Ca^{2+}$ remaining in the cells under isotopic equilibrium conditions

To test the role of intracellular stores in $^{45}Ca^{2+}$ release stimulated by adenosine, cellular $^{45}Ca^{2+}$ content under isotopic

Table 5. Effect of adenosine and various agonists on $^{45}Ca^{2+}$ content in mesangial cells under conditions of isotopic equilibrium

Cells were incubated in the presence of the agonists for 5 min under isotopic equilibrium conditions, i.e. in $^{45}Ca^{2+}$-loaded mesangial cells and in the presence of the isotope. Data are means ± S.E.M. The numbers of experiments are shown in parentheses; each experiment was done in triplicate or quadruplicate. Significant differences (*$P < 0.05$) versus control values and significant differences (†$P < 0.05$) versus values from cells treated with adenosine [one-way analysis of variance and multiple means comparison test (Scheffé test)] are shown.

Agonist	$^{45}Ca^{2+}$ content (d.p.m./mg of protein)
Control	24213 ± 978 (6)
KCl (60 mM)	21148 ± 1060* (5)
Adenosine (0.1 mM)	18622 ± 885* (6)
AVP (10 nM)	14549 ± 1086*† (5)
A23187 (10 μM)	12765 ± 1040*† (7)

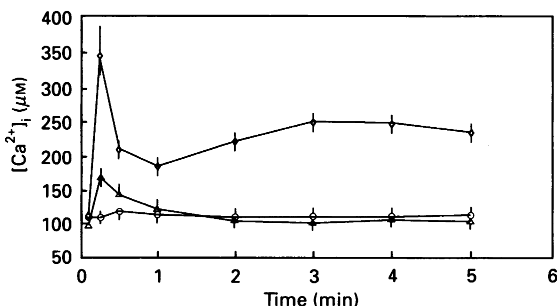

Fig. 2. Changes in $[Ca^{2+}]_i$ induced by 0.1 μM-adenosine (◇) or adenosine + 10 μM-verapamil (△) in cultured mesangial cells preloaded with fura-2

○, Control cells. Further details are given in the text. Data are means ± S.E.M.

equilibrium conditions was studied. Under these conditions the intracellular isotope content is an indicator of the total $[Ca^{2+}]_i$. After 5 min of incubation, adenosine induced a 23% decrease in the $^{45}Ca^{2+}$ content of the cultures whereas Ionophore A23187 (10 μM) decreased it by 50% under these conditions. AVP (0.1 μM), which has been shown to mobilize Ca^{2+} from intracellular stores, induced about a 40% decrease in $^{45}Ca^{2+}$ content with respect to the basal isotope content. Treatment of the cells with 60 mM-KCl also resulted in a slight decrease (15%) in intracellular radioactivity (Table 5). The net depletion of intracellular Ca^{2+} induced by all of these agents excludes the possibility of an increased $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange as a cause of the stimulation of $^{45}Ca^{2+}$ release elicited by them.

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

Adenosine-induced increases in $[Ca^{2+}]_i$ had two components, one very fast and the other very slow, with two peaks of $[Ca^{2+}]_i$, the first being of higher magnitude. After addition of adenosine the rapid elevation in $[Ca^{2+}]_i$ occurred within 15 s. The $[Ca^{2+}]_i$ decreased to near-basal values after 1 min, and then it increased again, reaching another maximum between 3 and 4 min (Fig. 2). The time course of the changes in $[Ca^{2+}]_i$ after adenosine treatment was biphasic, and rather similar to that reported for other vasoactive agents in mesangial cells [25,26], characterized by a rapid and transient increase on $[Ca^{2+}]_i$ and a sustained phase with a $[Ca^{2+}]_i$ above basal values. When cells were pretreated with verapamil, the peak increase in $[Ca^{2+}]_i$ was markedly attenuated, and the second, slow, phase of the response was not observed (Fig. 2).

DISCUSSION

In this study we demonstrate that adenosine, at the concentration which induces the contraction of cultured rat mesangial cells [16], stimulates Ca^{2+} movements into and out of mesangial cells and increases the $[Ca^{2+}]_i$.

There are some lines of evidence indicating that adenosine receptors can modulate Ca^{2+} channels. It has been reported that adenosine or its analogues decrease transmembrane flux into the cells in guinea pig atria [8,27], isoprenaline-stimulated mammalian ventricular myocytes [28], K^+-depolarized rat aortic smooth muscle cells [29], bovine coronary arteries [30] and brain cells [12]. In the kidney, adenosine operates in a different way to what has been reported in other tissues, since it induces renal vasoconstriction [14,31], not vasodilation as in other vascular beds [6]. The renal vasoconstriction as well as the glomerular and mesangial cell contraction induced by adenosine seem to depend on Ca^{2+} influx through potential-operated Ca^{2+} channels [14–16]. Our results shown that adenosine stimulates Ca^{2+} uptake in mesangial cells through verapamil-sensitive Ca^{2+} channels to an extent similar to that observed in KCl-depolarized mesangial cells. Although we can not exclude the possibility of an interaction between adenosine and verapamil at the receptor level, as has been described for dihydropyridine Ca^{2+} channel antagonists [9], this is unlikely because of the very low affinity of adenosine receptors for verapamil [32]. The increase in Ca^{2+} uptake induced by PIA, a selective agonist of the A_1-subtype adenosine receptor, the lack of effect of NECA, a selective agonist of the A_2-subtype adenosine receptor, as well as the blockade of the adenosine-induced increase in ^{45}Ca uptake by a selective antagonist of the A_1-subtype receptor (PD116, 948), strongly suggest that this effect is mediated by a A_1-type adenosine receptor.

In addition, adenosine stimulates $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded mesangial cells. This Ca^{2+} release is accompanied by a net depletion of the intracellular $^{45}Ca^{2+}$ content when the cells are under isotopic equilibrium conditions, which suggests that the $^{45}Ca^{2+}$ efflux stimulated by adenosine is the consequence of a mobilization of Ca^{2+} from intracellular stores.

It has been demonstrated that several hormonal substances with contractile effects in mesangial cells, such as endothelin, AVP and angiotensin II, cause both Ca^{2+} release from intracellular pools and stimulation of phosphoinositide turnover [18,19,25,33]. With respect to adenosine, preliminary results from our laboratory have shown that adenosine at 0.1 mM increased total inositol phosphate content and stimulated InsP_3 formation within 15 s in mesangial cells [34]. It has been reported that adenosine and its analogues stimulate phosphoinositide turnover and increase cytosolic Ca^{2+} in rabbit renal cortical collecting tubule cells in culture [1,35] and in rat tumour mast cells [10]. Further support for a possible involvement of an InsP_3-triggered mechanism in mediating the adenosine-stimulated Ca^{2+} efflux, is as follows: (1) the transient peak in $[Ca^{2+}]_i$ induced by adenosine is not different from that mediated by vasoactive peptides and other substances whose action on Ca^{2+} release is mediated by InsP_3 [18,19,36], and (2) TMB-8, a relatively selective intracellular Ca^{2+}-release blocker [37], partially inhibits the adenosine-induced $^{45}Ca^{2+}$ efflux from mesangial cells. Although the mechanism of action of TMB-8 is still unknown, this

compound inhibits transient Ca^{2+} responses that are accompanied by increases in InsP_3 in different cell types [38–41].

However, it is also possible that the Ca^{2+} efflux is not only due to a mobilization of Ca^{2+} from InsP_3-sensitive stores. We have tested the possibility that a rapid Ca^{2+} flux in the cells could trigger Ca^{2+} release from internal Ca^{2+} pools, by studying the effect of depolarization with KCl on mesangial cell Ca^{2+} fluxes. KCl is known to stimulate voltage-operated Ca^{2+} channels [24,42]. Our results show that, in mesangial cells, KCl induces a verapamil-inhibitable stimulation of $^{45}Ca^{2+}$ uptake and an increase in $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded mesangial cells. This Ca^{2+} release cannot be explained in terms of a stimulation of $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange, because under isotopic equilibrium conditions KCl produced a slight but significant decrease in the $^{45}Ca^{2+}$ content of the mesangial cells. This suggests a KCl-induced Ca^{2+} liberation from intracellular stores. The effect of KCl on Ca^{2+} release, like that induced by adenosine, seems to be based on Ca^{2+} entry, as it is blocked by verapamil. However, unlike the adenosine effect, it is not affected by TMB-8. Thus a mechanism, which is different from the TMB-8-inhibitable release of Ca^{2+} from intracellular stores that occurs with most contractile agonists, could be involved. The rapid Ca^{2+} exchange induced by KCl may be the result of a 'Ca^{2+}-induced Ca^{2+} release'-like mechanism [43] caused by the depolarization and a rapid increase in Ca^{2+} influx or by a depolarization of internal membranes and exchange of Cl^- for Ca^{2+} [44].

The mechanism of Ca^{2+} release from intracellular stores induced by adenosine in these cells is probably a combination of the two mechanisms described above, i.e. an InsP_3-mediated Ca^{2+} release (like that demonstrated for some other hormones) and a Ca^{2+}-entry-dependent Ca^{2+} release (as shown for KCl). In accordance with this hypothesis, when the cells were pretreated with TMB-8 the adenosine-induced ^{45}Ca-efflux curve became superposable with the curve obtained with KCl or KCl+TMB-8, but total inhibition was not observed. On the other hand, the depletion of intracellular $^{45}Ca^{2+}$ content induced by adenosine was less than that evoked by AVP, a hormone that mobilizes Ca^{2+} by an InsP_3-mediated mechanism, and greater than that evoked by cell depolarization with KCl.

Another important aspect to consider is that, whatever the mechanism mediating mobilization of Ca^{2+} from $^{45}Ca^{2+}$-loaded cells, it is dependent on extracellular Ca^{2+}, since it does not occur in Ca^{2+}-free medium. It also seems to be dependent on Ca^{2+} influx, since it is inhibited in the presence of verapamil. The Ca^{2+}-influx-dependent $^{45}Ca^{2+}$ efflux does not exclude any of the proposed mechanisms. On the one hand, $^{45}Ca^{2+}$ efflux stimulated by KCl is also blocked by verapamil. On the other hand, although Ca^{2+} entry is not necessary for phospholipase C activation, Ca^{2+}-dependent stimulation of phospholipase C has been described in some cases [45,46].

The action of adenosine on Ca^{2+} movements from and into mesangial cells is accompanied by an elevation of $[Ca^{2+}]_i$. The time course of the changes in $[Ca^{2+}]_i$ induced by adenosine showed an initial transient peak, as described for vasoactive peptides and other substances in mesangial cells [25,26], and a secondary increase which was slower and of less magnitude than the initial peak. A similar curve was described for angiotensin II in mesangial cells, and the two phases of the $[Ca^{2+}]_i$ increase seemed to be the result of different mechanisms [25]. In support of the previous contention, the initial transient peak of $[Ca^{2+}]_i$ induced by adenosine was partially blunted by verapamil, whereas the slow, secondary, increase was completely blocked by verapamil.

An interesting subject for further investigation is to determine the type of purinergic receptor involved in all these responses. It has been suggested that a P_1-adenosine receptor, different from the A_1- and A_2-type adenosine receptors, is involved in the increase in $[Ca^{2+}]_i$ induced by adenosine and its analogues in different cell types [11,14]. Our results demonstrate that the activation of the A_1-subtype adenosine receptor in mesangial cells induces an increase in ^{45}Ca uptake, thus suggesting that adenosine-induced Ca^{2+} uptake may also be mediated by a P_1-adenosine receptor. Although we cannot discount the involvement of a P_2-purinergic receptor, as reported for ATP in mesangial cells [17], this is not very likely owing to the low affinity of ATP (P_2) receptors for adenosine [47].

In summary, the present results demonstrate that, in cultured mesangial cells, adenosine induced an increase in $[Ca^{2+}]_i$, and stimulated Ca^{2+} influx and Ca^{2+}-influx-dependent Ca^{2+} efflux from intracellular stores. The adenosine-stimulated Ca^{2+} efflux has two components, a TMB-8-inhibitable Ca^{2+} release from intracellular stores and a TMB-8-non-inhibitable release. The mechanism of the Ca^{2+} release cannot be conclusively defined by the present results, but it seems to be the consequence of both an InsP_3-dependent mechanism and an InsP_3-independent one, similar to that produced by depolarization with KCl.

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REFERENCES

1. Londos, C., Cooper, D. M. F. & Wolff, J. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2551–2554
2. Fain, J. N. & Malbon, C. C. (1979) Mol. Cell. Biochem. **25**, 143–169
3. Van Calker, D., Müller, M. & Hamprecht, B. (1979) J. Neurochem. **33**, 999–1005
4. Marquardt, D. L. & Walker, L. L. (1988) Biochem. Pharmacol. **37**, 4019–4025
5. Delahunty, M., Cronin, J. & Linden, J. (1988) Biochem. J. **255**, 69–77
6. Berne, R. M. (1980) Circ. Res. **47**, 807–813
7. Brückner, R., Fenner, A., Meyer, W., Nobis, T. M., Schmitz, W. & Sholz, H. (1985) J. Pharmacol. Exp. Ther. **234**, 766–774
8. Cerbai, E., Klöckner, U. & Isenberg, G. (1988) Am. J. Physiol. **255**, H872–H878
9. Cheung, W. T., Shi, M. M., Young, J. D. & Lee, C. M. (1987) Biochem. Pharmacol. **36**, 2183–2186
10. Chin, J. H. & DeLorenzo, R. J. (1985) Brain Res. **348**, 381–386
11. Arend, L. J., Burnatowska-Hledin, M. A. & Spielman W. S. (1988) Am. J. Physiol. **255**, C581–C588
12. Ribeiro, J. A. & Sebastiao, A. M. (1986) Prog. Neurobiol. **26**, 179–209
13. Ali, H., Cunha-Melo, J. R., Saul, W. F. & Beaven M. A. (1990) J. Biol. Chem. **265**, 745–753
14. Macias, J. F., García, C., Santos, J. C., Sanz, E. & López-Novoa, J. M. (1985) J. Lab. Clin. Med. **106**, 562–567
15. Rossi, N., Churchil, P., Ellis, V. & Amore, B. (1988) Am. J. Physiol. **255**, H885–H890
16. Olivera, A., Lamas, S., Rodriguez-Puyol, D. & López-Novoa, J. M. (1989) Kidney Int. **35**, 1300–1305
17. Pfeilschifter, J. (1989) Eur. J. Clin. Invest. **19**, 347–361
18. Pfeilschifter, J. (1990) Cell. Signalling **2**, 129–138
19. Dunlop, M. E. & Larkins, R. G. (1991) Biochem. J. **272**, 561–568
20. Rodriguez-Puyol, D., Lamas, S., Olivera, A., López-Farré, A., Ortega, G., Hernando, L. & López-Novoa, J. M. (1989) Kidney Int. **35**, 632–638
21. Grynkiewicz, G., Poenie, M. & Tsien, R. (1985) J. Biol. Chem. **260**, 3440–3550
22. Lopez-Rivas, A. & Rozengurt, E. (1983) Biochem. Biophys. Res. Commun. **114**, 240–247
23. Borle, A. B. & Studer, R. (1978) J. Membr. Biol. **38**, 51–72

24. Goldfrain, T., Miller, R. & Wibo, M. (1986) Pharmacol. Rev. **38**, 321–416
25. Hassid, A., Pidikiti, N. & Gamen, D. (1986) Am. J. Physiol. **251**, F1018–F1028
26. Bonventre, J. V., Weber, P. C. & Gronich, J. H. (1988) Am. J. Physiol. **254**, F87–F94
27. De Biasi, M., Froldi, G., Ragazzi, E., Pandolfo, L., Caparrotta, L. & Fassina, G. (1989) Br. J. Pharmacol. **97**, 866-872
28. Isenberg, G. & Belardinelli, L. (1984) Circ. Res. **55**, 309–325
29. Fenton, R. A., Bruttig, S. P., Rubio, R. & Berne, R. M. (1982) Am. J. Physiol. **242**, H797–H804
30. Ramagopal, M. V. & Mustafa, S. J. (1988) Am. J. Physiol. **255**, H1492–H1498
31. Churchill, P. C. & Bidani, A. (1987) Am. J. Physiol. **252**, F299–F303
32. Marangos, P. J., Deckert, J. & Bisserbe, J. C. (1987) in Topics and Perspectives in Adenosine Research (Gerlach, E. & Becker, B. F., eds.), pp. 74–88, Springer Verlag, Berlin
33. Simonson, M. S., Wann, S., Mene, P., Dubyak, G. R., Kester, M., Nakazato, Y., Sedor, J. R. & Dunn, M. J. (1989) J. Clin. Invest. **83**, 708–712
34. Olivera, A., López-Rivas, A., Lamas, S., Rodriguez-Puyol, D. & López-Novoa, J. M. (1989) Kidney Int. **35**, 314 (Abstract)
35. Arend, L. J., Handler, J. S., Rhim, J. S., Gusovsky, F. & Spielman, W. S. (1989) Am. J. Physiol. **256**, F1067–F1074
36. Mene, P., Simonson, M. S. & Dunn, M. J. (1989) Physiol. Rev. **69**, 1347–1424
37. Chiou, C. Y. & Malagodi, M. H. (1975) Br. J. Pharmacol. **53**, 279–285
38. Ganz, M. B., Pekar, S. K., Perfetto, M. C. & Sterzel, R. B. (1988) Am. J. Physiol. **255**, F898–F906
39. Renard, D., Petit-Koskas, E., Génot, E., Dugas, B., Poggioli, J. & Kolb, J. P. (1988) Eur. J. Immunol. **18**, 1705–1711
40. Abbolian, T. E. (1989) Am. J. Physiol. **256**, F1135–F1143
41. Fuse, I. & Tai, H. H. (1988) Biochim. Biophys. Acta **972**, 54–59
42. Yu, Y. M., Lermioglu, F. & Hassid, A. (1989) Am. J. Physiol. **257**, F1094–F1099
43. Wakui, M., Osipchuk, Y. V. & Petersen, O. H. (1990) Cell **63**, 1025–1032
44. Martonosi, A. N. (1984) Physiol. Rev. **64**, 1240–1320
45. Farese, R. (1988) Am. J. Med. Sci. **296**, 223–230
46. Rana, R. S. & Hokin, L. E. (1990) Physiol. Rev. **70**, 115–164
47. Nees, S., DesRosiers, C. & Böck, M. (1987) in Topics and Perspectives in Adenosine Research (Gerlach, E. & Becker, B. F., eds.), pp. 454–467, Springer Verlag, Berlin

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