Effect of adenosine and adenosine analogues on cyclic AMP accumulation in cultured mesangial cells and isolated glomeruli of the rat

Ana Olivera & ¹*Jose M. Lopez–Novoa

Fundación Jimenez Diaz–Consejo Superior de Investigaciones Científicas, Madrid and *Departamento de Fisiología y Farmacología, Universidad de Salamanca, Salamanca, Spain

1 Changes in intracellular levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) were studied in rat isolated glomeruli and cultured glomerular mesangial cells exposed to adenosine and to the preferential A₁ receptor agonist N⁶-R-1-methyl-2-phenylethyl adenosine (R-PIA), or the potent A₂ adenosine receptor agonist 5-(N-ethylcarboxamide)adenosine (NECA).

2 Whereas NECA and adenosine triggered a dose-dependent increase in cyclic AMP values with EC₅₀ values of approximately 10⁻⁶ M and 3 × 10⁻⁵ M respectively, R-PIA lowered cyclic AMP levels at concentrations of 10⁻⁶ M or less and increased them at higher concentrations.

3 The time-course of the increase induced by 10⁻⁶ M NECA was slower than that induced by 10⁻⁴ M adenosine. Adenosine produced a maximal stimulation within the first minute, whereas the effect of NECA in both glomeruli and mesangial cells was noticeable only from the second minute of incubation.

4 The effects of the agonists R-PIA and NECA on the cyclic AMP system were blocked respectively by the A₁ adenosine receptor antagonist, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) at 10⁻⁶ M and the A₂ antagonist N-(2-dimethylaminoethyl)-N-methyl-4-(2, 3, 6, 7-tetrahydro-2, b-dioxo-1, 3-dipropyl-1H-purin-8-yl) benzene sulphonamide (PD115,199) at 10⁻⁶ M. Theophylline, a known antagonist of adenosine receptors, inhibited the action of adenosine on cyclic AMP in mesangial cells. Dipyridamole, an inhibitor of the uptake of adenosine by the cells, enhanced the response to adenosine.

5 These results suggest the existence of A₁ and A₂ adenosine receptors with opposite actions on intracellular levels of cyclic AMP in both glomeruli and mesangial cells. Adenosine seems to increase cyclic AMP through the activation of a surface adenosine receptor with pharmacological properties distinct from those exhibited by the A₂ adenosine receptor.

Keywords: Adenosine; adenosine receptors; cyclic AMP; glomeruli; mesangial cells; NECA; RPIA; kidney; xanthines

Introduction

Adenosine modulates important kidney functions, mainly local blood flow (Spielman *et al.*, 1980), renin secretion (Murray & Churchill, 1985) and glomerular filtration rate (GFR) (Osswald *et al.*, 1978). The two main types of adenosine receptor related to the adenylate cyclase system, A₁ and A₂, have been localized or characterized in several kidney structures that are involved in the regulation of these functions, i.e. the renal microvasculature (Freissmuth *et al.*, 1987), juxtaglomerular cells (Churchill & Churchill, 1985) and glomeruli (Abboud & Dousa, 1983).

Glomeruli possess A₁ and A₂ receptors (Freissmuth *et al.*, 1987) and respond to adenosine with an increase in the accumulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Abboud & Dousa, 1983), although the distribution of these receptors on the different types of glomerular cells and the ability of each population to respond to adenosine remain unexplored. Intraglomerular mesangial cells seem to be important in the control of GFR by virtue of their contractile properties (Kon & Ichikawa, 1985) and we have previously shown that adenosine causes these cells and isolated glomeruli to contract (Lopez–Novoa *et al.*, 1987; Olivera *et al.*, 1989). This suggests a role for mesangial cells in the reduction of the GFR induced by adenosine.

In the present study we have set out to identify functional A₁ and A₂ receptors in glomerular mesangial cells and isolated glomeruli by studying the effects of the relatively

selective adenosine agonists N⁶-R-1-methyl-2-phenylethyl-adenosine (R-PIA) (A₁) and 5-(N-ethylcarboxamide)adenosine (NECA) (A₂) on the intracellular content of cyclic AMP. We also investigated the effect of adenosine on the intracellular accumulation of cyclic AMP in mesangial cells.

Methods

Glomerular isolation and mesangial cell culture

Glomeruli were isolated from Wistar rats weighing 200 to 250 g by successive mechanical sieving (105 and 75 µm) as previously described (Rodríguez–Puyol *et al.*, 1986). The final preparation consisted of glomeruli without Bowman's capsule and without afferent or efferent arterioles. Tubular contamination was less than 5%.

For mesangial cell culture, glomeruli isolated from Wistar rats weighing 150 to 200 g by successive mechanical sieving (150 and 50 µm), were treated with collagenase (300 u ml⁻¹), plated in 35 mm plastic tissue culture dishes (Costar) and maintained in the conditions previously described (Olivera *et al.*, 1989; Rodríguez–Puyol *et al.*, 1989). The culture medium consisted of RPMI 1640 supplemented with 10% foetal calf serum, L-glutamine (1 mM), penicillin (0.66 µg ml⁻¹), streptomycin sulphate (60 µg ml⁻¹), and buffered with HEPES, pH 7.2. The culture medium was changed every two days. Studies were performed on day 21 or 22, at which time epithelial cells were no longer detected in the culture dishes. The identity of the cells was confirmed by morphological and functional criteria previously described (Olivera *et al.*, 1989; Rodríguez–Puyol *et al.*, 1989).

¹ Author for correspondence at: Jose M. Lopez–Novoa, Dept. of Physiology and Pharmacology, Faculty of Medicine, Avda. Campo Charro s/n, 37007 Salamanca, Spain.

Intracellular and intraglomerular cyclic AMP determination

Cells were incubated for 5 min in Tris-glucose buffer (Composition mM: Tris 20, NaCl 130, KCl 10, sodium acetate 10 and glucose 5, pH 7.4) with 2.5 mM CaCl_2 . The buffer was then aspirated and replaced by the same buffer containing adenosine or the adenosine analogues. At the times indicated, the medium was aspirated, and the cells washed twice with ice-cold buffer. Intracellular cyclic AMP was extracted twice with 1.0 ml absolute ethanol. Cells were scraped from the dishes and protein measured by the method of Lowry *et al.* (1951). In the experiments in which forskolin was used to stimulate adenylate cyclase, the preincubation and incubation buffers also contained 5 μM forskolin. When adenosine antagonists or dipyrindamole were used, they were also present in the preincubation and incubation buffers.

Isolated glomeruli were resuspended in ice-cold Tris-glucose buffer containing 2.5 mM CaCl_2 . Five aliquots of 50 μl were taken from this pool for protein determination, and samples of 500 μl were added to polystyrene tubes and kept in ice. The glomerular suspension was placed in a shaking water bath at 37°C for 20 min. After this preincubation period, 500 μl of the same buffer containing the substances to be tested was added to the suspension. The incubation was terminated at different times (1, 2, 5 and 30 min) by adding 2 ml of ice-cold buffer and centrifuging for 30 s in a microfuge at 1000 g. Supernatants were aspirated and replaced by 1 ml of absolute ethanol. The ethanolic extraction of intraglomerular cyclic AMP was performed twice.

Ethanolic extracts from each sample were pooled and evaporated in a stream of nitrogen. Dried samples were resuspended and cyclic AMP was assayed with a commercial kit. The recovery of cyclic AMP during the extraction procedure, determined as percentage of recovery of [^3H]-cyclic AMP added to the sample (2000–3000 c.p.m.), was greater than 89% in all cases. The inter- and intra-assay coefficients of variation were 11.9% and 8.4% respectively. Cross-reactivities with the adenosine and adenosine agonists and antagonists used were: adenosine, 10^{-4} M: 0.00151%; R-PIA, 10^{-6} M: 0.0066%; NECA 10^{-6} M: 0.0061%; 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) 10^{-6} M: 0.0036%; N2(2-dimethylaminoethyl) - N - methyl - 4 - (2,3,6,7 - tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)benzenesulphonamide PD115,199 10^{-6} M: 0.0073% ($n = 5-7$).

Materials

Adenosine, R-PIA (N^6 -R-1-methyl-2-phenylethyl adenosine), NECA (5-(N-ethylcarboxamide)adenosine), theophylline (1,3-dimethylxanthine), forskolin (7-acetoxy-8,13-epoxy-1 ϕ ,6 β , 9-trihydroxylabd-14-ene-11-one), collagenase type 1A from *Clostridium histolyticum*, and L-glutamine were purchased from Sigma (St Louis, MO, U.S.A.). DPCPX (8-cyclopentyl-1,3-dipropylxanthine) and PD115,199 (N-(2-dimethylaminoethyl) -N-methyl-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) benzenesulphonamide) were kind gifts from Warner-Lambert, Parke Davis, Ann Arbor, Michigan, U.S.A. Adenosine deaminase was purchased from Boehringer Mannheim GmbH, Germany. Dipyrindamole (Persantin) and isoprenaline (Aleudrin) were from Boehringer Ingelheim, Barcelona, Spain. Penicillin was obtained from Laboratorios Level SA, Barcelona, Spain. Streptomycin sulphate was obtained from Antibioticos SA, Madrid, Spain and amphotericin B from Squibb Industria Farmaceutica S.A., Barcelona, Spain. RPMI 1640, Hanks balanced salt solution and foetal calf serum were obtained from Flow Laboratories, Woodcock Hill, UK. The [^{125}I]-cyclic AMP (single range) assay system was purchased from Amersham.

Statistics

Values are given as mean $\pm$ s.e. mean. Statistical differences between means were assessed either by the Kruskal-Wallis test or by one way/two way analysis of variance, followed by Scheffé's test. $P < 0.05$ was considered to be statistically significant.

Results

Effects of adenosine and adenosine analogues on cyclic AMP accumulation

Adenosine induced a concentration-dependent increase in the intracellular cyclic AMP content in mesangial cells with an EC_{50} of approximately 3×10^{-5} M, that was statistically significant from unstimulated values in the supramicromolar range (Figure 1). The potent A_2 adenosine analogue, NECA, also induced a concentration-dependent increase in cyclic AMP, with an EC_{50} of approximately 10^{-6} M. The relatively selective A_1 agonist R-PIA, had a dual effect, i.e. it decreased cyclic AMP content at concentrations lower than 10^{-6} M, but caused an increase at concentrations above 10^{-6} M.

To test for possible endogenous adenosine synthesis in mesangial cells that might explain the relatively high doses needed to obtain a response, the cells were preincubated for 10 min in the presence of adenosine deaminase (2 u ml^{-1}) to inactivate endogenous adenosine before washing and incubating with adenosine for 5 min. The pretreatment with adenosine deaminase did not significantly modify basal cyclic AMP levels (basal: 11.4 ± 0.8 , $n = 18$; adenosine deaminase: 14.7 ± 3.6 pmol mg^{-1} protein; $n = 7$) or the effect of adenosine 10^{-4} M (adenosine: 25.3 ± 5.4 , $n = 12$; adenosine + adenosine deaminase: 28.0 ± 7.1 pmol mg^{-1} protein; $n = 7$).

In subsequent experiments adenosine was used at 10^{-4} M, a concentration giving a maximal response and the concentration used in previous studies (Olivera *et al.*, 1989). Adenosine analogues were used at 10^{-6} M, at which concentration R-PIA caused contraction of mesangial cells (unpublished results).

Time course changes in cyclic AMP levels

The time-courses of changes in cyclic AMP content induced by the agonists and adenosine in isolated glomeruli and

Figure 1 Concentration-response curves for adenosine (■), NECA (□) and R-PIA (▼) on intracellular cyclic AMP accumulation in mesangial cells after 5 min incubation. Values are mean with s.e.mean (vertical lines) of 7–12 experiments. Asterisks represent significant differences ($P < 0.05$ or higher, Scheffé's test), compared with basal (●).

untreated mesangial cells, and in cells prestimulated with forskolin $5 \mu\text{M}$, are shown in Figures 2, 3 and 4, respectively. In mesangial cells, adenosine induced an increase in cyclic AMP within the first minute of stimulation that was maintained for at least 5 min, before falling to basal levels by 10 min (Figure 3). However, levels of cyclic AMP increased again after 15 min incubation with adenosine and remained elevated up to 30 min (Control: 9.8 ± 1.2 , $n = 9$; adenosine: $16.2 \pm 4.5 \text{ pmol mg}^{-1} \text{ protein}$, $n = 13$). In glomeruli, a slight and statistically insignificant enhancement of intraglomerular cyclic AMP remained after 30 min of incubation (9.5 ± 0.1 , $n = 7$ versus $6.4 \pm 0.9 \text{ pmol mg}^{-1} \text{ protein}$ in control conditions, $n = 10$). The rapid response to adenosine (within 1 min) occurred both in isolated glomeruli (Figure 2) and in forskolin - prestimulated and untreated mesangial cells (Figures 3 and 4), but the increase in cyclic AMP induced by NECA in these preparations was considerably slower (Figures 2, 3 and 4). In contrast to the effect of adenosine or NECA, the A_1 agonist R-PIA either had no significant effect

or reduced the intracellular cyclic AMP content in isolated glomeruli and mesangial cells. When the agonists R-PIA and NECA were added together, each at 0.5 or $1 \times 10^{-6} \text{ M}$, both to isolated glomeruli and cultured mesangial cells, the response was similar to that induced by adenosine (data not shown).

Effect of adenosine A_1 and A_2 receptor blockade

Neither R-PIA nor NECA is completely subtype specific. To establish that their observed actions were mediated by A_1 and A_2 receptors respectively, the effects of adding the potent A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and the A_2 antagonist PD115,199 both at 10^{-6} M , were examined. The increase in cyclic AMP induced by NECA was blocked by the relatively selective A_2 antagonist, but not by the A_1 antagonist, whereas the A_1 antagonist blocked the inhibitory effect of R-PIA in forskolin-pretreated cells (Figure 5). The antagonists alone had no effect on cyclic AMP content.

Figure 2 Time-course of changes in intraglomerular cyclic AMP induced by 10^{-6} M adenosine (■), 10^{-6} M R-PIA (▼) and 10^{-6} M NECA (□). Data are expressed as a percentage over basal cyclic AMP at each time. Values are mean with s.e.mean (vertical lines) of 8–15 experiments. Asterisks represent significant differences ($P < 0.05$ or higher, Scheffé test), compared with control values.

Figure 3 Time-course of changes in intracellular cyclic AMP content induced by 10^{-6} M adenosine (■), 10^{-6} M R-PIA (▼) or 10^{-6} M NECA (□) in glomerular mesangial cells. Values are mean with s.e.mean (vertical lines) of 9–18 experiments. Asterisks represent significant differences ($P < 0.05$ or higher, Scheffé test), compared with control values (●).

Figure 4 Time-course of changes in intracellular cyclic AMP content induced by adenosine analogues in forskolin-treated mesangial cells. Treatments are 10^{-4} M adenosine (■), 10^{-6} M R-PIA (▼), 10^{-6} M NECA (□) or 10^{-6} M NECA + DPCPX (○). Values are mean with s.e.mean (vertical lines) of 6–18 experiments. Asterisks represent significant differences ($P < 0.05$ or higher, Scheffé test), compared with control values (●).

Figure 5 Effects of the relatively selective antagonists PD115,199 (AT_2 , 10^{-6} M) and DPCPX (AT_1 , 10^{-6} M) on the changes in cyclic AMP induced by 10^{-6} M R-PIA or 10^{-6} M NECA in mesangial cells. Cells were preincubated in the presence of forskolin (F, $5 \mu\text{M}$) for 5 min and incubated with the agonists for 2 min. Values are mean with s.e.mean (vertical lines) of 4–8 experiments. Asterisks represent significant differences ($P < 0.05$ or higher, Scheffé test), compared with control values.

To determine the type of adenosine receptor involved in the response to adenosine, the effects of the same antagonists were tested. Neither of the antagonists modified the stimulation of cyclic AMP production by adenosine in either forskolin pretreated or untreated mesangial cells (Figure 6) or in isolated glomeruli (results not shown). Only when both antagonists were used together, each at 5×10^{-7} M, was an inhibition of the effect of adenosine observed. A similar result was obtained when both antagonists were used together at a concentration of 5×10^{-6} M each. The inhibition was partial in cells pretreated with forskolin and almost maximal (not significantly different from the inhibition induced by theophylline) in non-pretreated cells (Figure 6).

Effects of theophylline and dipyridamole

To demonstrate further that the effect of adenosine is mediated by a specific membrane receptor and not by an intracellular receptor or by the action of products of adenosine metabolism, we studied the action of theophylline and dipyridamole on the stimulation of cyclic AMP accumulation by adenosine. Theophylline, a methylxanthine used as an adenosine receptor antagonist, inhibited the increase in cyclic AMP induced by adenosine (Figure 7). This effect was specific for adenosine receptors since theophylline at the same concentration (10^{-4} M) failed to inhibit the increase in cyclic AMP induced by isoprenaline (10^{-4} M) (control: 11.4 ± 0.8 , $n = 18$; isoprenaline: 32.0 ± 6.6 , $n = 7$; $P < 0.01$; isoprenaline + theophylline: 35.6 ± 3.8 pmol mg $^{-1}$ protein, $n = 7$; $P < 0.01$ versus control). On the other hand, dipyridamole, a drug that inhibits the transport of adenosine into the cells, potentiated the increase in cyclic AMP induced by adenosine in mesangial cells (adenosine alone: $122 \pm 19\%$ $n = 12$; dipyridamole + adenosine: $159 \pm 15\%$, $n = 13$, $P < 0.05$, by the Kruskal-Wallis test). Theophylline at 10^{-4} M did not significantly modify intracellular cyclic AMP (Figure 7) but dipyridamole used alone augmented cyclic AMP con-

Figure 6 Effects of the relatively selective A_2 antagonist PD115,199 (AT_2 , 10^{-6} M) or A_1 antagonist DPCP (AT_1 , 10^{-6} M) or both ($AT_1 + AT_2$, 10^{-6} M) on the increase in cyclic AMP induced by 10^{-4} M adenosine (Aden) in mesangial cells in the presence (hatched columns, right ordinate scale) or absence (open columns, left ordinate scale) of forskolin. Forskolin-treated cells were preincubated in the presence of forskolin ($5 \mu\text{M}$) for 5 min and incubated with adenosine for 2 min. Values are mean with s.e.mean (vertical lines) of 5–8 experiments. Asterisks represent significant differences ($P < 0.05$ or higher, Scheffé test), compared with control values (Con). Circles represent significant differences ($P < 0.05$ or higher, Scheffé test) compared with adenosine.

Figure 7 Effect of the adenosine receptor antagonist theophylline (Theo, 10^{-4} M) on the stimulatory response to 10^{-4} M adenosine (Aden) on intracellular cyclic AMP accumulation in mesangial cells. Values are mean with s.e.mean (vertical lines) of 7–18 experiments.

Asterisks represent significant differences ($P < 0.01$, Kruskal-Wallis test) versus control values (Con).

tent over basal values (control: 11.4 ± 0.8 , $n = 18$; dipyridamole: 25.2 ± 1.3 pmol mg $^{-1}$ protein, $n = 6$, $P < 0.01$).

Discussion

While there have been some previous *in vivo* and *in vitro* studies of the effects of adenosine and adenosine analogues on glomeruli and glomerular function (Osswald *et al.*, 1978; Abboud & Dousa, 1983; Hall *et al.*, 1985; Freissmuth *et al.*, 1978; Lopez-Novoa *et al.*, 1987) no studies have been reported on the effects of these compounds on different types of glomerular cell. Thus the primary purpose of this work was to study in glomerular mesangial cells the effect of adenosine and its analogues on intracellular cyclic AMP, one of the most important messengers mediating the effects of adenosine (Londos *et al.*, 1980; Shimizu, 1983) and involved in regulating cellular responses (Ross & Gilman, 1980).

The results presented here suggest the existence of both A_1 and A_2 adenosine receptors on mesangial cells and in that respect complement information already reported for whole glomeruli. One of the widely accepted criteria for the demonstration of distinct adenosine receptor subtypes has been the differential effects obtained with relatively selective adenosine agonists (Londos *et al.*, 1980; Daly, 1982; Burns *et al.*, 1987a). We used R-PIA as a relatively selective A_1 agonist (Daly, 1982) and NECA as a potent A_2 adenosine agonist, although it also binds to the A_1 receptor (Bruns *et al.*, 1987a; Daly, 1982). Adenosine and NECA raised cyclic AMP levels in mesangial cells and isolated glomeruli, whereas R-PIA at doses up to 10^{-6} M decreased cyclic AMP content in both mesangial cells and glomeruli. The rank-order of potency for the increase in cyclic AMP (NECA > adenosine > R-PIA) is the one expected for an A_2 -mediated response, whereas the inhibitory effect of R-PIA suggests the existence of classical A_1 or R_1 (Londos *et al.*, 1980) adenosine receptors in glomeruli and mesangial cells. The dose-response effect of R-PIA was biphasic because of its ability to bind to A_2 receptors at high concentrations. The A_1 receptor, which has been shown to be of high affinity (Londos *et al.*, 1980; Bruns *et al.*, 1987a), was activated at low doses of R-PIA, and the A_2 receptor, which is supposed to be of low affinity, was activated at high doses of R-PIA. When the basal activity of the adenylate cyclase was enhanced by

forskolin, the inhibitory effect of R-PIA was more evident. Forskolin pretreatment was used in some of the experiments because the slight changes that may be induced by different compounds are more easily observed when cyclase activity is enhanced.

Further evidence of the involvement of A_1 and A_2 adenosine receptor subtypes in the inhibitory (R-PIA) and stimulatory (NECA) responses, comes from the fact that the highly selective A_1 adenosine antagonist DPCPX (PD116,948, Haleen *et al.*, 1987), inhibited the effect of R-PIA on cyclic AMP, but not that of NECA, in forskolin-pretreated mesangial cells. However, the potent A_2 antagonist PD115,119 (Bruns *et al.*, 1987b) blocked the stimulatory effect of NECA, but not the inhibitory effect of R-PIA.

Adenosine, like the agonist NECA, increased cyclic AMP accumulation in isolated glomeruli and mesangial cells. The increase in cyclic AMP levels induced by adenosine in mesangial cells was insufficient to reach statistical significance at micromolar concentrations. The relatively high doses necessary to obtain a response with adenosine cannot be explained in terms of endogenous synthesis of adenosine by the cultures since treatment with adenosine deaminase affected neither basal values of cyclic AMP nor the stimulatory action of adenosine on intracellular cyclic AMP content. Both adenosine-stimulated and basal cyclic AMP levels in the glomeruli were very similar to those reported by Abboud & Dousa (1983). Although the contribution of epithelial and endothelial glomerular cells to the adenosine-induced rise in intraglomerular cyclic AMP has not been studied, mesangial cells seem to be the main source of cyclic AMP in the glomerulus (Mene *et al.*, 1989).

In theory, adenosine could cause an increase in cyclic AMP levels either through a stimulatory coupling to adenylate cyclase or an inhibitory action on cyclic AMP breakdown (Shenolikar, 1988). Several studies have provided evidence that adenosine acts via coupling of its receptor to adenylate cyclase rather than by an action on cyclic AMP phosphodiesterase (Londos *et al.*, 1980; Shimizu, 1983; Arend *et al.*, 1987; Burnatowska-Hledin & Spielman, 1991). In isolated glomeruli, adenosine appeared to act by stimulating adenylate cyclase (Abboud & Dousa, 1983). From our results in mesangial cells we cannot discount the possibility of an action of adenosine on the phosphodiesterase. However, the effect of dipyrindamole, a compound that inhibits adenosine transport into the cells, seems to support the interpretation of an action of adenosine via a surface receptor coupled to adenylate cyclase. Dipyrindamole has been shown to inhibit cyclic AMP phosphodiesterase in some cells (Abboud & Dousa, 1983; Schoeffter *et al.*, 1987), and increases basal levels of cyclic AMP in glomeruli (Abboud & Dousa, 1983) and in our mesangial cell cultures. The enhancement by dipyrindamole of the stimulatory action of adenosine could be the result of the inhibition of cyclic AMP phosphodiesterase by this compound. Nevertheless, the existence of an inhibitory intracellular P site for high doses of adenosine (Londos *et al.*, 1980; Garcia-Sainz & Torner, 1985) in mesangial cells cannot be ruled out, and dipyrindamole, by blocking adenosine transport, may prevent access of adenosine to this site.

The present results demonstrate the involvement of a specific surface receptor for adenosine. First, theophylline, a known inhibitor of the interaction of adenosine with its surface receptors (Schwabe *et al.*, 1985), inhibits the stimulatory effect of adenosine on glomerular (Abboud & Dousa, 1983) and mesangial cell content of cyclic AMP.

Secondly, the addition of dipyrindamole to inhibit adenosine uptake into the cell, potentiates the increase induced by adenosine in intraglomerular (Abboud & Dousa, 1983) or intracellular cyclic AMP. These results argue strongly against an action of adenosine as a precursor for intracellular ATP synthesis, the substrate for cyclic AMP formation, and also against the possible mediation of products of intracellular adenosine deamination elicited by adenosine.

The receptor involved in the response to adenosine in these glomerular cells seems to exhibit properties different from those described for A_1 and A_2 receptors. First, the relatively selective antagonists PD115,199 and DPCPX when used separately, failed to inhibit the response to adenosine either in glomeruli or mesangial cells whether or not they were preincubated with forskolin, and only when the antagonists were added together, did they block the adenosine response. Secondly, the stimulatory effect of adenosine was observed 1 min earlier than the effect of NECA. The stimulation of cyclic AMP described in other cells for adenosine or adenosine analogues is not usually observed before 2 min of incubation and it is usually maximal from 5 to 30 min (Van Calcar *et al.*, 1979; Jonzon *et al.*, 1985; Arend *et al.*, 1987; Gatti *et al.*, 1988). In mesangial cells and isolated glomeruli, NECA showed the typical stimulation time-course, but the effect of adenosine was consistently faster. These results suggest that the action of adenosine on cyclic AMP is characteristic of neither A_1 nor A_2 adenosine receptor subtypes. Adenosine surface receptors with unusual pharmacological and physiological properties have already been described in several cell types (Chin & DeLorenzo, 1986; Arend *et al.*, 1988; Belloni *et al.*, 1989; Ali *et al.*, 1990), and these receptors have sometimes been called A_3 receptors (Ribeiro & Sebastiao, 1986). An alternative explanation could be the existence in mesangial cells of two types of A_2 receptor, A_{2a} and A_{2b} . The A_{2b} , the low affinity A_2 adenosine receptor, has been described only in brain and fibroblasts, and cannot bind the A_2 antagonist PD115,199 (Bruns *et al.*, 1987b). In addition, it is possible that the occupation of both A_1 and A_2 receptors by high doses of adenosine produces a synergistic interaction similar to that described for α - and β -adrenoreceptors in parathyroid cells (McKinney *et al.*, 1989). This latter hypothesis is supported by the fact that when the agonists R-PIA and NECA were used together at 0.5 or 1×10^{-6} M each, both in isolated glomeruli and cultured mesangial cells, the response was neither like that to R-PIA nor like that to NECA, but was similar to that induced by adenosine.

In summary, our findings demonstrate that both A_1 and A_2 adenosine receptors are present in glomeruli and in cultured glomerular mesangial cells. The results with the selective antagonists and the different time-courses of cyclic AMP accumulation produced by adenosine and its agonist analogues suggest that the effect of adenosine may not be mediated solely by activation of an A_2 -like adenosine receptor.

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References

- ABBOUD, H.E., & DOUSA, T.P. (1983). Action of adenosine on cyclic 3',5'-nucleotides in glomeruli. *Am. J. Physiol.*, **244**, F633-F638.
- ALI, H., CUNHA-MELO, J.R., SAUL, W.F., & BEAVEN, M.A. (1990). Activation of phospholipase C via adenosine receptors provides synergistic signals for secretion in antigen-stimulated RBL-2H3 cells. Evidence for a novel adenosine receptor. *J. Clin. Invest.*, **265**, 715-753.
- AREND, L.J., SONNENBURG, W.K., SMITH, W.L. & SPIELMAN, W.S. (1987). A₁ and A₂ adenosine receptors in rabbit cortical collecting tubule cells, modulation of hormone-stimulated cAMP. *J. Clin. Invest.*, **79**, 710-714.
- AREND, L.J., BURNATOWSKA-HLEDIN, M.A. & SPIELMAN, W.S. (1988). Adenosine receptor-mediated calcium mobilization in cortical collecting tubule cells. *Am. J. Physiol.*, **255**, C581-C588.
- BURNATOWSKA-HLEDIN, M.A. & SPIELMAN, W.S. (1991). Effects of adenosine on cAMP production and cytosolic Ca²⁺ in cultured rabbit medullary thick limb cells. *Am. J. Physiol.*, **260**, C143-C150.
- BELLONI, F.L., BELARDINELLI, L., HALPERIN, C. & HINTZE, T.H. (1989). An unusual receptor mediates adenosine-induced SA nodal bradycardia in dogs. *Am. J. Physiol.*, **256**, H1553-H1564.
- BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1987a). Adenosine receptor subtypes: binding studies. In *Topics and Perspectives in Adenosine Research*. ed. Gerlach, E. & Becker, B.F. pp.59-73. Berlin: Springer-Verlag.
- BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A. & HAYS, S.J. (1987b). PD 115,199: an antagonist ligand for adenosine A₂ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **335**, 64-69.
- CHIN, J.H. & DELORENZO, R.J. (1986). A new class of adenosine receptors in brain. Characterization by 2-Chloro[³H]-adenosine binding. *Biochem. Pharmacol.*, **35**, 847-856.
- CHURCHILL, P.C. & CHURCHILL, M.C. (1985). A₁ and A₂ adenosine receptor activation inhibits and stimulates renin secretion of rat renal cortical slices. *J. Pharmacol. Exp. Ther.*, **232**, 589-594.
- DALY, J.W. (1982). Adenosine receptors: targets for future drugs. *J. Med. Chem.*, **25**, 197-207.
- FREISSMUTH, M., HAUSLEITHNER, V., TUISL, E., NANOFF, C. & SCHUTZ, W. (1987). Glomeruli and microvessels of the rabbit kidney contain both A₁ and A₂ adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **335**, 438-441.
- GARCIA-SAINZ, J.A. & TORNER, M.L. (1985). Rat fat cells have three types of adenosine receptors (Ra, Ri and P). Differential effects of pertussis toxin. *Biochem. J.*, **232**, 439-443.
- GATTI, G., MADEDDU, L., PANDIELLA, A., POZZAN, T. & MALDONESI, J. (1988). Second-messenger generation in PC12 cells. Interactions between cyclic AMP and Ca²⁺ signals. *Biochem. J.*, **255**, 753-760.
- HALEEN, S.J., STEFFEN, R.P. & HAMILTON, H.W. (1987). PD 116,948, a highly selective A₁ adenosine receptor antagonist. *Life Sci.*, **40**, 555-561.
- HALL, J.E., GRANGER, J.P. & HESTER, R.L. (1985). Interactions between adenosine and angiotensin II in controlling glomerular filtration. *Am. J. Physiol.*, **248**, F340-F346.
- JONZON, B., NILSSON, J. & FREDHOLM, B.B. (1985). Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. *J. Cell Physiol.*, **124**, 441-456.
- KON, V. & ICHIKAWA, I. (1985). Hormonal regulation of glomerular filtration. *Annu. Rev. Med.*, **36**, 515-531.
- LONDOS, C., COOPER, D.M. & WOLFF, J. (1980). Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2251-2254.
- LOPEZ-NOVOA, J.M., ARRIBA, G., BARRIO, V. & RODRIGUEZ-PUYOL, D. (1987). Adenosine induces a calcium-dependent glomerular contraction. *Eur. J. Pharmacol.*, **134**, 365-367.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
- MCKINNEY, J.S., DESOLE, M.S. & RUBIN, R.P. (1989). Convergence of cAMP and phosphoinositide pathways during rat parotid secretion. *Am. J. Physiol.*, **257**, C651-C657.
- MENE, P., SIMONSON, M.S. & DUNN, M.J. (1989). Physiology of the mesangial cell. *Physiol. Rev.*, **69**, 1347-1424.
- MURRAY, R.D. & CHURCHILL, P.C. (1985). Concentration dependency of the renal vascular and renin secretory response to adenosine receptor agonists. *J. Pharmacol. Exp. Ther.*, **232**, 189-193.
- OLIVERA, A., LAMAS, S., RODRIGUEZ-PUYOL, D. & LOPEZ-NOVOA, J.M. (1989). Adenosine induces mesangial cell contraction by an A₁-type receptor. *Kidney Int.*, **35**, 1300-1305.
- OSSWALD, H., SPIELMAN, W.S. & KNOX, F.G. (1978). Mechanisms of adenosine-mediated decreases in glomerular filtration rate in dogs. *Circ. Res.*, **43**, 465-469.
- RIBEIRO, J.A. & SEBASTIAO, A.M. (1986). Adenosine receptors and calcium: basis for proposing a third (A₃) class adenosine receptor. *Prog. Neurobiol.*, **26**, 179-209.
- RODRIGUEZ-PUYOL, D., ARRIBA, G., BLANCHART, A., SANTOS, J.C., CAMELO, C., FERNANDEZ-CRUZ, A., HERNANDO, L. & LOPEZ-NOVOA, J.M. (1986). Lack of a direct regulatory effect of ANF on prostaglandins and renin release by isolated rat glomeruli. *Biochem. Biophys. Res. Commun.*, **138**, 496-501.
- RODRIGUEZ-PUYOL, D., LAMAS, S., OLIVERA, A., LOPEZ-FARRE, A., ORTEGA, G., HERNANDO, L. & LOPEZ-NOVOA, J.M. (1989). Actions of Cyclosporine A on cultured rat mesangial cells. *Kidney Int.*, **35**, 632-637.
- ROSS, E.M. & GILMAN, A. (1980). Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.*, **49**, 533-564.
- SCHOEFFTER, P., LUGNIER, C., DEMESY-WAELEDE, F. & STOCLET, J.C. (1987). Role of cyclic AMP- and cyclic GMP-phosphodiesterases in the control of cyclic nucleotide levels and smooth muscle tone in rat isolated aorta. A study with selective inhibitors. *Biochem. Pharmacol.*, **36**, 3965-3972.
- SCHWABE, U., UKENA, D. & LOHSE, M.J. (1985). Xanthine derivatives as antagonists at A₁ and A₂ adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **330**, 212-221.
- SHENOLIKAR, S. (1988). Protein phosphorylation: hormones, drugs, and bioregulation. *FASEB J.*, **2**, 2753-2764.
- SHIMIZU, H. (1983). Adenosine receptors associated with the adenylate cyclase system. In *Physiology and Pharmacology of Adenosine Derivates*. ed. Daly, J.W., Kuroda, Y., Phillis, J.W., Shimizu, H. & Ui, M. pp.31-40. New York: Raven Press.
- SPIELMAN, W.S., BRITTON, S.L. & FIKSEN-OLSEN, M.J. (1980). Effect of adenosine on the distribution of renal blood flow in dogs. *Circ. Res.*, **46**, 449-456.
- VAN CALKER, D., MÜLLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999-1005.

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