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Ca^{2+} dependence of the response of three adenosine type receptors in rat hepatocytes

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Abstract

The effect of three different receptor-specific adenosine agonists on the rate of ureagenesis by isolated rat hepatocytes and the dependence on the external free Ca^{2+} concentration ($[Ca^{2+}]_e$) were investigated. In the presence of high $[Ca^{2+}]_e$ all adenosine receptor agonists increased ureagenesis to similar levels. However, with low $[Ca^{2+}]_e$ the effects of each agonist varied as follows: (i) the adenosine A_1 receptor agonist, 2-chloro-N^6-cyclopentyl-adenosine, increased ureagenesis depending partially on $[Ca^{2+}]_e$, (ii) the adenosine receptor A_2 agonist, 2-*p*-(-2-carboxy-ethyl) phenethylamino-5′-*N*-ethylcarboxyamido adenosine hydrochloride, increased ureagenesis independently of $[Ca^{2+}]_e$ and (iii) in contrast, the adenosine receptor A_3 agonist N^6-2-(-4-aminophenyl) ethyladenosine, increased ureagenesis only in the presence of high $[Ca^{2+}]_e$. The adenosine receptor A_1 antagonist, 1-allyl-3,7-dimethyl-8-phenyl xanthine, inhibited the effect of the adenosine receptor A_1 agonist on ureagenesis, but not the effect of the adenosine A_2 or A_3 receptor agonists. The adenosine A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine, inhibited only the effect of the adenosine A_2 receptor agonist. Thus, in addition to A_1 and A_2 type adenosine receptors, rat hepatocytes possess an A_3-like adenosine receptor which responds to the addition of an adenosine A_3 agonist by accelerating ureagenesis a $[Ca^{2+}]_e$ dependent manner. Moreover, it was observed that in the presence of extracellular Ca^{2+} each agonist increased $[Ca^{2+}]_i$ and this effect was inhibited by the appropriate specific antagonist. © 1997 Elsevier Science B.V.

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1. Introduction

In mammals, the interaction of adenosine with the different adenosine receptors found in the plasma membrane of each type of cell results in a large number of physiological effects. There are at least five subtypes of adenosine receptors. These have been classified as: A_1, A_{2A}, A_{2B}, A_3 and A_4. This classification is supported by biochemical, pharmacological and molecular cloning evidence (Dalziel and Westfall, 1994; Palmer et al., 1995).

In some organs and species, such as mouse and humans, adenosine A_3 receptors have been cloned. However, no reports are available on the physiological response of the adenosine A_3 receptor in the liver. The synthesis of adenosine receptor-specific agonists allows the analysis of the cellular response to the stimulation of each specific adenosine receptor type. Thus, in this paper, the effects of three adenosine receptor-specific agonists and two adenosine receptor-specific antagonists on the rate of urea synthesis in isolated rat hepatocytes are reported. The dependence of the ureagenic response on $[Ca^{2+}]_e$ was investigated in order to gain insight into the transduction signal(s) involved for each of these receptors within the liver.

2. Materials and methods

All reagents were of the highest purity available commercially. Adenosine, quin-2, A23187, bovine serum albumin type V and dimethylsulfoxide were from Sigma Co., St Louis, MO. Adenosine agonists were chosen according to their affinity for each kind of receptor (RBI catalog, RBI Co, Natick, MA). These were as follows: the adenosine A_1 receptor agonist was 2-chloro-N^6-cyclopentyl-adenosine (CCPA) ($K_a = 0.2–0.4$ nM); the adenosine A_2

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receptor agonist was 2-*p*-(-2-carboxy-ethyl) phenethylamino-5′-*N*-ethylcarboxyamido adenosine hydrochloride (CGS-21680) ($K_a = 15$ nM) and the adenosine A_3 receptor agonist was N^6-2-(-4-aminophenyl) ethyladenosine (APNEA) ($K_a = 15.5$ nM). Two adenosine receptor-specific antagonists were used: the adenosine A_{1r} receptor antagonist was 1-allyl-3,7-dimethyl-8-phenyl xanthine (ADMPX) ($K_i = 0.6$ nM) and the adenosine A_2 receptor antagonist was 3,7-dimethyl-1-propargylxanthine (DMPX) ($K_i = 11$ μM).

Hepatocytes were isolated from male Wistar rats weighing 150 to 200 g and starved for 24 h by the method described by Berry and Friend (1969), except we used Ringer–Krebs medium without calcium and then added calcium with the collagenase, instead of using calcium-free Krebs–Henseleit (Guinzberg et al., 1987). In addition, we added 1% bovine serum albumin to the Ringer–Krebs medium used for washing the isolated cells (Guinzberg et al., 1987). Isolated hepatocytes were incubated in Krebs–Ringer-bicarbonate/1% bovine serum albumin, pH 7.4, at 37°C and under aeration with 95%/5% oxygen/carbon dioxide. Hepatocytes were used within 1 h of preparation.

Urea synthesis was determined after 60 min (Gutman and Bergmeyer, 1974) in Krebs-bicarbonate medium supplemented with 10 mM glucose, 5 mM ammonium carbonate and 3 mM ornithine. Where indicated, 1.2 mM $CaCl_2$ or 1.2 mM EGTA was added 20 min prior to incubation.

Cytoplasmic calcium concentrations ($[Ca^{2+}]_i$) were measured by using the metallochromic dye Quin-2 as described before (Charest et al., 1983; Díaz et al., 1991). Briefly, isolated hepatocytes were incubated in Krebs–Ringer bicarbonate, to approximately 50 mg wet weight/ml, under a saturated 95%/5% oxygen/CO_2 atmosphere and at 37°C. After 10 min, 100 μM Quin-2/AM was added from a 20 mM stock solution in dimethylsulfoxide. After 20 min, cells were washed by centrifuging them twice at 500 rpm/3 min in a clinical centrifuge. Liver cells were distributed in 200 μl aliquots in Eppendorf tubes and immersed in ice until used (Díaz et al., 1991). Fluorescence was measured in a Farrand Mark II spectrofluorometer at 340–500 nm, using 10 nm slits. At the end of each trace, fluorescence was calibrated in order to determine the $[Ca^{2+}]_i$ under each experimental condition. Maximal fluorescence was determined by adding 10 μM ionomycin to the cells and allowing $[Ca^{2+}]_e$ to equilibrate, saturating Quin-2. Then minimal fluorescence was determined by adding 0.1% Triton X-100 to disrupt the cells and 100 μM $MnCl_2$ to quench the fluorescence of Quin-2. Experimental $[Ca^{2+}]_i$ was calculated as described by Tsien et al. (1982).

3. Results

In the presence of 1.2 mM $[Ca^{2+}]_e$ all adenosine receptor agonists produced a dose-dependent increase in the rate of ureagenesis (Figs. 1–3). Ureagenesis increased to about the same extent after the addition of each agonist. The rate of ureagenesis after the addition of the adenosine agonist

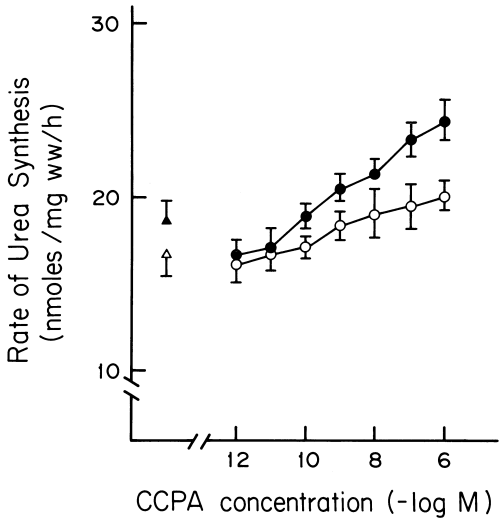

Fig. 1. Effects of different concentrations of the adenosine A_1 receptor agonist, 2-chloro-N^6-cyclopentyl-adenosine (CCPA), on the rate of urea synthesis. Isolated hepatocytes (35 mg wet wt) were suspended in 1 ml of Krebs–Ringer medium with either (●) 1.2 mM Ca^{2+} or (○) 1.2 mM EGTA. Control urea synthesis was measured in the absence of agonist with (▲) or without (△) Ca^{2+}. Results are expressed as means ± S.E.M (bars) of duplicate incubations from four cell preparations. In the presence of 1.2 mM $[Ca^{2+}]_e$, the response to 10^{-10} M CCPA was significant at $P < 0.05$ in a Student's *t*-test. From 10^{-9} to 10^{-6} M CCPA, $P < 0.01$.

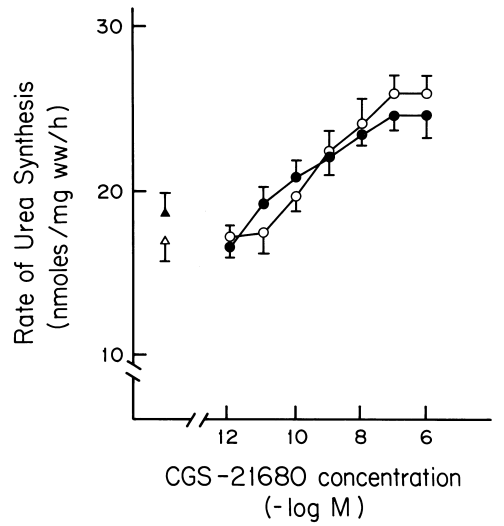

Fig. 2. Effects of different concentrations of the adenosine A_2 receptor agonist, 2-*p*(-2-carboxy-ethyl)phenethylamino-5′-*N*-ethylcarboxyamido adenosine hydrochloride (CGS-21680), on the rate of urea synthesis. Experimental conditions as in Fig. 1, (●) 1.2 mM Ca^{2+}, (○) 1.2 mM EGTA. Control urea synthesis was measured in the absence of agonist with (▲) or without (△) Ca^{2+}. Results are expressed as means ± S.E.M (bars) of duplicate incubations from four cell preparations. The response to 10^{-10} M to 10^{-6} M CGS-21680 was significant at $P < 0.05$ in Student's *t*-test.

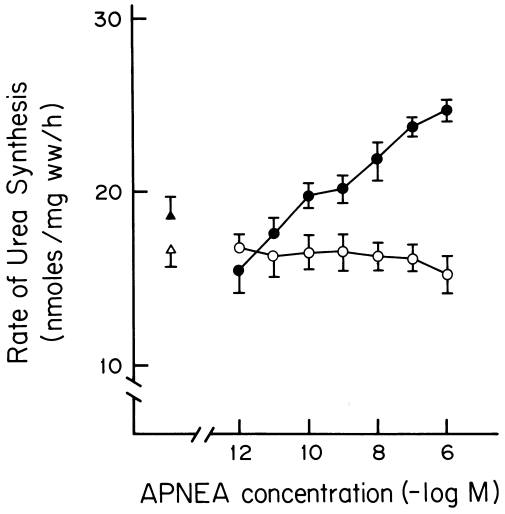

Fig. 3. Effects of different concentrations of the adenosine A_3 receptor agonist, N^6-2-(-4-aminophenyl) ethyladenosine (APNEA), on the rate of urea synthesis. Experimental conditions as in Fig. 1. (●) 1.2 mM Ca^{2+}, (○) 1.2 mM EGTA. Control urea synthesis was measured in the absence of agonist with (▲) or without (△) Ca^{2+}. Results are expressed as means ± S.E.M (bars) of duplicate incubations from four cell preparations. In the presence of 1.2 mM $[Ca^{2+}]_e$, the response to 10^{-11} and 10^{-10} M APNEA was significant at $P < 0.05$ in Student's *t*-test. From 10^{-9} at 10^{-6} M APNEA, $P < 0.01$.

was similar to the rate observed previously in the presence of adenosine (Guinzberg et al., 1993). In the presence of the adenosine A_1 and A_2 receptor agonists, a significant stimulation was detected at adenosine agonist concentrations of 10^{-10} M ($P < 0.05$) and increased with concentrations up to 10^{-6} M ($P < 0.01$). The adenosine A_3 receptor agonist APNEA, stimulated ureagenesis at 10^{-11} M ($P < 0.05$) and this stimulation increased with agonist concentrations up to 10^{-6} M APNEA ($P < 0.01$). APNEA stimulated ureagenesis in spite of reports indicating that rat hepatocytes do not contain adenosine A_3 receptors (Linden, 1994).

In order to assess the dependence on $[Ca^{2+}]_e$ of the response elicited by each adenosine receptor agonist, the same experiments were conducted in the absence of $[Ca^{2+}]_e$. All adenosine receptor agonists were used at a final concentration of 1 μM. It was observed that the ureagenic response to each adenosine receptor agonist was different when Ca^{2+} was not added to the incubation medium. In the absence of $[Ca^{2+}]_e$, the response to the adenosine receptor agonist A_1 was smaller (Fig. 1) and the response to agonist A_2 was the same that in the presence of $[Ca^{2+}]_e$ (Fig. 2), whereas the ureagenic response to the adenosine A_3 receptor agonist was totally absent if $[Ca^{2+}]_e$ was low (Fig. 3).

To further define the involvement of each adenosine receptor on the ureagenic response, two adenosine receptor-specific antagonists were used to titrate the effects of each adenosine receptor agonist (Fig. 4A). Increasing concentrations of the adenosine A_1 receptor antagonist inhibited the response to the adenosine A_1 receptor agonist while the responses to the adenosine A_2 and A_3 receptor agonists were not affected (Fig. 4A). The adenosine A_2 receptor antagonist inhibited the response to the adenosine A_2 receptor agonist, but did not inhibit the adenosine A_1 or A_3 receptor agonist-mediated stimulation of ureagenesis (Fig. 4B).

Since the $[Ca^{2+}]_e$ dependence of the ureagenic response to each adenosine receptor agonist was different, it was decided to test the effects of each of the adenosine receptor agonists on $[Ca^{2+}]_i$ (Table 1). In contrast to the effects on ureagenesis, the effects on $[Ca^{2+}]_i$ of the adenosine recep-

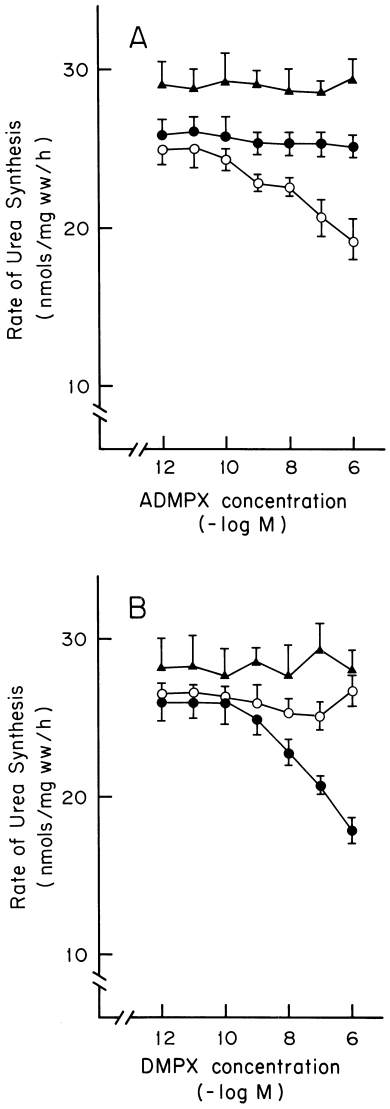

Fig. 4. Effects of two receptor-specific adenosine antagonists on the increased rate of ureagenesis produced by three specific receptor agonists. Experimental conditions as in Fig. 1, except each agonist was used at 10^{-6} M. (A) Increasing concentrations of the A_1 antagonist 1-Allyl 3-7 dimethyl-8 phenyl xanthine (ADMPX) or (B) increasing concentrations of the A_2 antagonist 3,7-dimethyl-1-propargylxanthine (DMPX). The agonists used were (○) the A1 agonist, CCPA, (●) the A_2 agonist, CGS-21680, (▲) the A_3 agonist, APNEA. Bars are S.E.M.

Table 1
Cytoplasmic Ca^{2+} concentrations, $[Ca^{2+}]_i$, detected in the presence and absence of different adenosine receptor agonists and antagonists

Agonist added	Control	A_1 antagonist	A_2 antagonist
None	57 ± 1.5		
A_1	75 ± 4.4, $P < 0.05$[a]	58 ± 4.7, $P < 0.05$[b]	74 ± 5.5
A_2	90 ± 4.3, $P < 0.01$[a]	91 ± 3.6	58 ± 2.2, $P < 0.01$[b]
A_3	109 ± 5.8, $P < 0.001$[a]	94 ± 3.1	94 ± 5.1
Adenosine	118 ± 3.4, $P < 0.001$[a]		

Experimental conditions as in Fig. 1. Agonists were: A_1, CCPA; A_2, CGS-21680 and A_3, APNEA. Antagonists were A_1, ADMPX; A_2, DMPX. All agonists and antagonists were used at 1 μM final concentration. Numbers are $[Ca^{2+}]_i$ in nM as means ± S.E.M. of duplicates from four cell preparations.
[a] Statistical significance, agonist versus control.
[b] Statistical significance, agonist plus antagonist versus agonist alone.

tor agonists were indistinguishable: in the presence of $[Ca^{2+}]_e$ all adenosine receptor agonists produced an increase in $[Ca^{2+}]_i$. In the absence of $[Ca^{2+}]_e$ none of the adenosine receptor agonists, nor adenosine, had any effect on $[Ca^{2+}]_i$ (results not shown). When the effects of the adenosine receptor antagonists were tested, the adenosine A_1 receptor antagonist ADMPX blocked the $[Ca^{2+}]_i$ increase produced by the adenosine A_1 receptor agonist but it did not inhibit the increase in $[Ca^{2+}]_i$ produced by the adenosine A_2 or A_3 receptor agonist. In contrast, the adenosine A_2 receptor antagonist DMPX inhibited the increase in $[Ca^{2+}]_i$ produced by the adenosine A_2 receptor agonist although it did not inhibit the effects of either the adenosine A_1 or A_3 receptor agonist (Table 1).

4. Discussion

The ureagenic response to three adenosine receptor agonists with different receptor type specificity was evaluated. In the presence of $[Ca^{2+}]_e$ each adenosine receptor agonist accelerated ureagenesis to the same extent. However, in the absence of $[Ca^{2+}]_e$ the response to each adenosine receptor agonist varied, suggesting that the mechanism by which each adenosine receptor mediated the increase in ureagenesis may not be the same. The large differences in the $[Ca^{2+}]_e$ dependence of the response to each of the adenosine receptor agonists suggest that the second messenger involved in the response to each adenosine receptor type varies, i.e. Adenosine A_3 receptors use Ca^{2+} as second messenger adenosine A_2 receptors use a second messenger different to Ca^{2+}, probably cAMP and adenosine A_1 receptors probably use a combination of second messengers, depending partially on Ca^{2+} for information relay. The participation of different second messengers in response to activation of each adenosine receptor has been documented, e.g. activation of adenylyl-cyclase via G_s protein and activation of Ca^{2+} channels via phospholipase C occur after stimulation of adenosine A_{2a} and A_{2b} receptors, respectively (Murthy et al., 1995; Palmer and Stiles, 1995; Hirano et al., 1996).

Adenosine A_3 receptors have not been detected in rat hepatocytes by using either specific antibodies or Northern blots (Linden, 1994). However, the adenosine A_3 agonist, APNEA was able to increase ureagenesis in rat hepatocytes. This response was not due to an interaction with either the adenosine A_1 receptor or the adenosine A_2 receptor because the addition of the specific antagonists was not inhibitory for the response to the adenosine A_3 receptor agonist. In contrast, the adenosine A_1 receptor-specific antagonist inhibited the response to the adenosine A_1 receptor agonist, and the adenosine A_2 receptor antagonist inhibited only the response to the adenosine A_2 receptor agonist. The same pattern of inhibition was observed when the increase in $[Ca^{2+}]_i$ produced by each adenosine receptor agonist was evaluated (Table 1). Further support for the existence of a third type of receptor in rat hepatocytes comes from the different $[Ca^{2+}]_e$ sensitivity of the ureagenic response to each agonist. The adenosine A_3 receptor agonist exhibited a strictly $[Ca^{2+}]_e$-dependent response. This was different to the effects of either the adenosine A_1 receptor, which was only partially dependent on $[Ca^{2+}]_e$, or the adenosine A_2 receptor agonist, which was independent of $[Ca^{2+}]_e$. In human liver, adenosine A_3 receptors have been identified by molecular cloning (Murrison et al., 1995). It is possible that rat hepatocytes may have a receptor which shares the same pharmacological specificity as adenosine A_3 receptors from other organs. The adenosine A_3 receptor from rat liver may be slightly different in structure to adenosine A_3 receptor subtypes described to date (Sajjadi et al., 1996), thus explaining why it is not detected with antibodies or Western blot systems (Linden, 1994). In this regard, small differences in the primary structure of adenosine receptors of a given subtype have been reported, which result in varying results for binding studies (Fredholm et al., 1994).

The different behavior of each adenosine receptor type would explain the conflicting results on the role that cAMP or Ca^{2+} may have as second messengers in adenosine-mediated processes in the cell (Marchand et al., 1979; Bartrons et al., 1984; Díaz et al., 1991; Guinzberg et al., 1993). It is interesting to note that the adenosine A_3 receptor agonist was the only agonist to exhibit the same $[Ca^{2+}]_e$ dependence as adenosine, i.e. it stimulated ureagenesis in the presence, but not in the absence, of $[Ca^{2+}]_e$ (Díaz et al., 1991).

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