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Effect of adenosine and inosine on ureagenesis in hepatocytes

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Adenosine and inosine produced a dose-dependent stimulation of ureagenesis in isolated rat hepatocytes. Hypoxanthine, xanthine and uric acid were without effect. Half-maximally effective concentrations were 0.08 μM for adenosine and 5 μM for inosine. Activation of ureagenesis by both nucleosides had the following characteristics: (*a*) it was observed with either glutamine or $(NH_4)_2CO_3$, provided that glucose was present; (*b*) it was not detected when glucose was replaced by lactate plus oleate; (*c*) it was mutually antagonized by glucagon, but not by adrenaline; and (*d*) it was dependent on Ca^{2+}. We suggest that the action of adenosine and inosine on ureagenesis might be of physiological significance.

INTRODUCTION

Our knowledge of the physiological and pathophysiological functions of the nucleoside adenosine has grown considerably. Among the described actions of the nucleoside on hepatic metabolism, it has been reported that adenosine increases ATP concentrations and the energy charge of the liver (Chagoya *et al.*, 1972). This is of interest, owing to the role of these metabolic indicators in the regulation of cell metabolism (Atkinson, 1977). Of even greater interest are the well-documented actions of adenosine as a hormone and as a neuromodulator (Burnstock, 1981; for a more recent review, see Stefanovich *et al.*, 1985). Considerable effort has been made to characterize adenosine receptors (Burnstock, 1981; Londos *et al.*, 1983), and to design more potent adenosine-receptor antagonists (Bruns *et al.*, 1983). Liver cells are in the growing list of cells containing adenosine receptors; isolated hepatocytes, on stimulation with adenosine, and at least two of its analogues, produce a response in the adenylate cyclase activity (Londos *et al.*, 1980). Furthermore, adenosine and adenine nucleotides cause dose-dependent activation of glycogen phosphorylase in isolated hepatocytes (Keppens & De Wulf, 1985). To increase our knowledge of adenosine actions, its role on urea biosynthesis was explored. Ureagenesis is one of the metabolic routes affected by numerous hormones, either dependent or not on modifications in the activity of adenylate cyclase (Corvera & García-Sáinz, 1982). Thus a response of ureagenesis to adenosine will give a more integrated picture of the actions of the nucleoside on the regulation of liver metabolism. Lund *et al.* (1975) found that adenosine added to isolated hepatocytes inhibits urea biosynthesis; a 0.5 mM dose of the nucleoside was required to produce maximal inhibition, this concentration being several orders of magnitude higher than the concentration of adenosine used as a hormone (Londos *et al.*, 1980). The present paper describes the response in urea biosynthesis by liver cells, incubated with low doses of adenosine or inosine within the range used to exert their role as hormones. Both nucleosides were tested alone or in combination with one of two well-known ureagenic hormones, glucagon and adrenaline (Titheradge & Haynes, 1980; Corvera & García-Sáinz, 1982).

MATERIALS AND METHODS

Male Wistar rats (150–200 g) starved 24 h before the beginning of the experiment were used, unless stated otherwise. Three different commercial diets were used (Table 1): initially, Purina rat chow imported from the United States (experiments reported in Fig. 1, Tables 2 and 4, and the ATP-pool assays); when the Mexican government forbade the importation of rat chow, the experiment was continued by feeding the rats with 'Nutricubos', made in Mexico (experiments reported in Table 3, and those with hepatocytes depleted of Ca^{2+}). Since the rate of urea biosynthesis was low with this diet, some control experiments were performed with a third diet, combining equal parts of 'Nutricubos' with 'Ladrina'; this combination produced (*a*) a better growth rate than with 'Nutricubos' alone, and (*b*) a growth rate identical with the optimal one reported by Lane (1971).

Hepatocytes were isolated by the method of Berry & Friend (1969), but the recommended Ca^{2+}-free Hanks

Table 1. Composition of commercial diets used

Values (%) are from the manufacturers' labels.

	Purina chow* 5001	Nutricubos Rodent Laboratory Chow diet†	Ladrina†
Crude protein not less than	23.0	23.0	27.0
Crude fat not less than	4.5	2.5	9.0
Crude fibre not more than	6.0	6.0	4.5
Ash not more than	8.0	8.0	10.0
Added minerals not more than	2.5	1.6	–
Nitrogen-free extract	–	48.5	39.5

* Ralston Purina Co., St. Louis, MO, U.S.A.
† Purina, S.A. de C.V. Km. 4.5 Carr. Constitución Querétaro, Qro. C.P. 76180 México.

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solution was replaced by Krebs–Ringer bicarbonate [Krebs & Henseleit (1932) prepared without Ca^{2+}] plus 10 mM-glucose (the latter was omitted in some experiments, as indicated below); the liver was perfused with these Ca^{2+}-free solutions; for the subsequent collagenase-digestion step, 1.2 mM-Ca^{2+} was added to the solution. In the first nine experiments, cell viability was measured by ATP contents and Trypan Blue exclusion. A value of 2.29 ± 0.32 μmol of ATP/g (mean ± S.E.M. for nine separate batches of cells) was obtained, which is comparable with the values found in the literature (e.g. Lund *et al.*, 1975); at least 90% of these cells excluded 0.2% Trypan Blue. Later, Trypan Blue exclusion was routinely assayed in all the experiments, and hepatocytes were used when their viability was above 85%.

The isolated cells were incubated under continuous shaking in Krebs–Ringer bicarbonate buffer in an atmosphere of O_2/CO_2 (19:1) for 60 min at 37 °C. The Krebs–Ringer buffer with 1.2 mM-Ca^{2+} was supplemented with 10 mM-glucose, 10 mM-L-glutamine, 3 mM-L-ornithine and 1% bovine serum albumin; the pH was adjusted to 7.4. In some experiments, as detailed in the Results section, 10 mM-glutamine was replaced with 10 mM-$(NH_4)_2CO_3$, and glucose by 10 mM-lactate plus 1 mM-oleate in the incubation medium. Furthermore, when cells were incubated with lactate + oleate, glucose was absent from the initial perfusion medium and the collagenase-digestion medium.

In a series of experiments, hepatocytes were dissociated in a Ca^{2+}-free medium supplemented with 1 mM-EGTA. These cells were incubated in Krebs–Ringer bicarbonate, free of Ca^{2+}, but supplemented with 10 mM-glucose, 10 mM-$(NH_4)_2CO_3$, 3 mM-L-ornithine and 1% bovine serum albumin.

Each assay was routinely performed in duplicate. Each tube contained 25–30 mg wet wt. of liver cells. At the end of the incubation period, the tubes were placed in an ice bath for 5 min, and then centrifuged at 50 *g* for 10 min. The supernatant was kept for urea quantification (Gutman & Bergmeyer, 1974). The numbers of observations given in Fig. 1 and the Tables, for any given experimental condition, refer to separate batches of cells.

ATP was quantified by the method of Lamprecht & Trautschold (1974). Adenosine, inosine, hypoxanthine, xanthine, uric acid, glucagon, L-adrenaline, L-glutamine, L-ornithine hydrochloride, urease (type III) and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase was from Worthington. Other reagents were analytical grade, from Merck, Mexico.

RESULTS

Dose–response curves (Fig. 1) yielded K_a values of 0.08 μM for adenosine and 5 μM for inosine on urea biosynthesis. Other metabolic derivatives of adenosine, i.e. hypoxanthine, xanthine and uric acid, did not show any ureagenic action (Table 2). The incubation mixture of these deaminated compounds was supplemented with 10 μM-NH_4Cl to stimulate the concentration of NH_4^+, obtained by exhaustive deamination of C-2 of adenosine, but identical results were recorded without this supplementation (not shown). The data obtained with adenosine, shown in Fig. 1 and Table 2, contrast with the inhibition of ureagenesis obtained in hepatocytes incubated with the same nucleoside (Lund *et al.*, 1975).

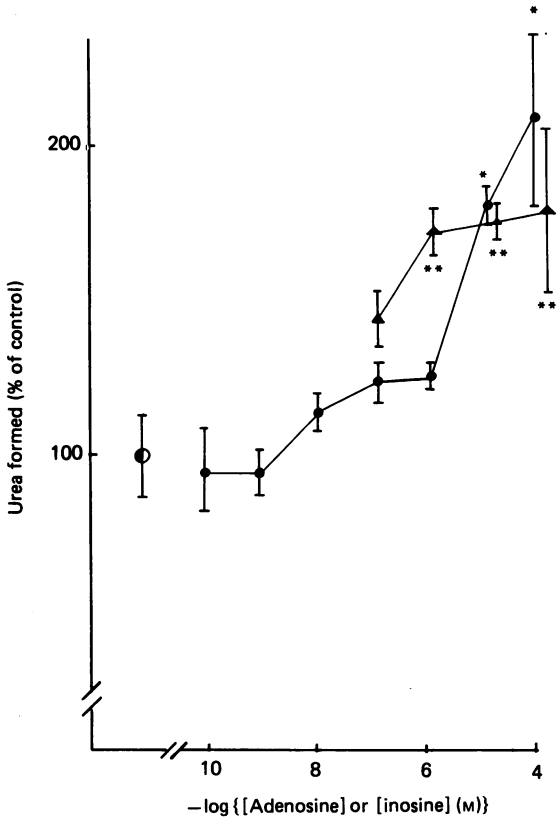

Fig. 1. Stimulation of urea synthesis by adenosine and inosine

Hepatocytes were incubated as described in the Materials and methods section: ◐, control; ●, with inosine; ▲, with adenosine. Results are expressed as percentages of the control value, which was 14.48 ± 3.12 nmol of urea formed/60 min per mg wet wt. Values plotted are the means, and vertical lines represent S.E.M., of duplicate incubations from four to six cell preparations, except the control sample, with 19 cell preparations. Statistical significance versus control is indicated: $*P < 0.05$, $**P < 0.001$.

In the experiments of Lund *et al.* (1975), 40 mg wet wt. of liver cells from 48 h-starved rats was incubated for 60 min at 37 °C with 10 mM-lactate, 1 mM-oleate, 10 mM-NH_4Cl and 2 mM-ornithine; their data show an inhibition of urea synthesis by adenosine, ranging from 23 to 65%, when the nucleoside was added at concentrations from 0.5 to 4.0 mM. Some experimental conditions used by those authors were assayed in the experiments shown in Table 3. The hepatocytes were incubated with 10 mM-lactate and 1 mM-oleate, instead of glucose, and with 10 mM-NH_4Cl or -$(NH_4)_2CO_3$ instead of glutamine; however, adenosine concentration was kept at 10 μM. The same ureagenic response to low concentrations of adenosine was obtained with glutamine, $(NH_4)_2CO_3$ (Table 3) and NH_4Cl (results not shown). Interestingly, replacement of glucose by lactate + oleate abolished the ureagenic effect of adenosine, with either glutamine or $(NH_4)_2CO_3$ (Table 3). Similar results (not shown) were obtained with 10 μM-inosine instead of adenosine.

One point requires additional information. The basal rate of urea synthesized was (in nmol/60 min per mg wet wt. of cells) 14.48 in Fig. 1 and Table 2, 7.60 in Table 3,

Table 2. Effect of adenosine catabolites on urea biosynthesis

Isolated hepatocytes were incubated as described in the Materials and methods section and supplemented with the concentrations of compounds indicated. Results are expressed as the means of (nmol of urea formed/60 min per mg wet wt. of cells) ± S.E.M. of duplicate samples from the numbers of preparations in parentheses. Statistical significance versus control is indicated.

No purine derivative No NH_4Cl	With 10 μM-adenosine No NH_4Cl	With 10 μM-inosine and 10 μM-NH_4Cl	With 10 μM-hypoxanthine and 10 μM-NH_4Cl	With 10 μM-xanthine and 10 μM-NH_4Cl	With 10 μM-uric acid and 10 μM-NH_4Cl
14.48 ± 3.12 (19)	25.16 ± 3.76 (3) $P < 0.001$	24.16 ± 2.76 (3) $P < 0.05$	15.00 ± 1.72 (3) $P < 0.9$	16.16 ± 2.74 (3) $P < 0.9$	16.04 ± 0.38 (3) $P < 0.9$

Table 3. Comparison of the ureagenic action of adenosine in cells incubated with different sources of oxidizable substrate and with different sources of ammonia

Hepatocytes were incubated in Krebs–Ringer bicarbonate, pH 7.4, supplemented with 3 mM-ornithine, 10 mM-glucose, or 10 mM-lactate plus 1 mM-oleate, 10 mM-glutamine, or 10 mM $(NH_4)_2CO_3$. Other conditions were as in Table 2.

Source of oxidizable substrate	Source of ammonia	Urea formed (nmol/60 min per mg wet wt. of cells)	
		No adenosine	+ 10 μM-adenosine
Glucose	Glutamine	7.60 ± 0.50 (5)	19.36 ± 2.64 (3) $P < 0.01$
Glucose	$(NH_4)_2CO_3$	7.42 ± 0.90 (15)	21.36 ± 0.20 (6) $P < 0.001$
Lactate + oleate	Glutamine	7.74 ± 1.00 (4)	6.50 ± 0.74 (4) $P < 0.05$
Lactate + oleate	$(NH_4)_2CO_3$	7.64 ± 1.46 (4)	6.28 ± 2.2 (3) $P < 0.9$

Table 4. Effects of adenosine or inosine on urea biosynthesis stimulated by glucagon and adrenaline

Isolated hepatocytes were incubated as described in the Materials and methods section with the indicated additions. Other conditions were as in Table 2.

Additions	Urea formed (nmol/60 min per mg wet wt. of cells)		
	No nucleoside	+ 100 μM-Adenosine	+ 10 μM-Inosine
No hormone	14.48 ± 3.12 (19)	25.38 ± 2.4 (10) $P < 0.001$*	24.16 ± 2.76 (3) $P < 0.05$*
Glucagon (1 μM)	43.46 ± 4.00 (7) $P < 0.001$*	7.74 ± 1.42 (4) $P < 0.001$* $P < 0.001$†	12.18 ± 1.40 (3) $P < 0.001$* $P < 0.05$†
Adrenaline (0.1 μM)	32.04 ± 2.20 (7) $P < 0.001$†	37.72 ± 2.00 (6) $P < 0.01$* $P < 0.1$†	24.12 ± 3.48 (3) $P < 0.9$* $P < 0.1$†

* Statistical significance versus control with nucleoside, but without hormone.
† Statistical significance versus control with hormone, but without nucleoside.

and 29.8 ± 1.0 ($n = 8$) in rats fed with the third diet (Table 1), a value similar to that found by Lund *et al.* (1975). In the last group of rats, adenosine produced a statistically significant dose-related stimulation, ranging from 14% at 0.1 μM to 25% at 10 μM, at which concentrations no increase in the ATP pool of isolated hepatocytes is observed. Thus the ability of adenosine to stimulate ureagenesis was observed with three different commercial diets.

To gain information on the mechanism involved in the ability of adenosine and inosine to stimulate ureagenesis, their responses were compared with those of glucagon and adrenaline; the possibility of additive effects was also explored (Table 4). A mixture of high doses of adenosine or inosine with glucagon mutually antagonized the effect of the nucleosides and that of the peptide hormone; however, the ureagenesis response to adrenaline was not modified by either adenosine nor inosine (Table 4).

Furthermore, stimulation of urea synthesis by both nucleosides (assayed at concentrations from 10 to 0.1 μM) was absent in cells depleted of Ca^{2+} by pretreatment with EGTA, and incubated in a Krebs–Ringer medium without Ca^{2+} (results not shown).

DISCUSSION

The results indicate that ureagenesis in the liver is controlled by some purine metabolites. Adenosine and inosine cause a dose-dependent activation of ureagenesis (Fig. 1). This ability is not shared by other catabolic products of these nucleosides (Table 2). The possibility that adenosine or inosine could have acted as ureagenesis substrates, thus explaining their stimulatory role, is ruled out on the following bases: (*a*) catabolic metabolites of inosine plus NH_4Cl were unable to stimulate urea synthesis (Table 2), and (*b*) assuming a 100% deamination of 10 μM-adenosine, the contribution of the NH_4^+ formed is negligible as compared with a 10 mM pool of NH_4^+, the supplemented substrate for urea synthesis (Table 3).

The fact that the stimulatory effect of adenosine and inosine was observed with glutamine and with $(NH_4)_2CO_3$ (Table 3) rules out the participation of glutamine in the mechanism involved in the regulatory steps going from NH_4^+ to urea.

Stimulation of ureagenesis by adenosine and by inosine is mediated by a Ca^{2+}-dependent mechanism. This conclusion is supported by the absence of any stimulation by adenosine or inosine of ureagenesis in Ca^{2+}-depleted hepatocytes. Additional, but circumstantial, evidence includes the report that ureagenesis is affected by other hormones, independently from modifications in the activity of adenylate cyclase (Corvera & García-Sáinz, 1982). However, a mutual antagonism between adenosine or inosine and glucagon, but not adrenaline, opens more possibilities to study the mechanism(s) involved in signal transduction for hormones, particularly in the regulation of ureagenesis, a metabolic route in which several links are missing between the initial stimulation of a receptor by its hormone and the final metabolic response.

Finally, the concentration of adenosine in rat liver cells is of the order of 10 μM (Ueland & Saeb, 1979), although 75% of the total tissue adenosine is bound to intracellular proteins (Belloni *et al.*, 1984). Therefore the results reported in the present paper are in the range of the physiological concentration of at least one of the nucleosides used in the study. It is suggested that the actions described here of adenosine and inosine in the regulation of urea biosynthesis might be of physiological relevance.

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