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Regulation of glycogen metabolism in hepatocytes through adenosine receptors. Role of Ca^{2+} and cAMP

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Abstract

The objective of this work is to identify the adenosine receptor subtype and the triggered events involved in the regulation of hepatic glycogen metabolism. Glycogenolysis, gluconeogenesis, cAMP, and cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) were measured in isolated hepatocytes challenged with adenosine A_1, A_{2A}, and A_3 receptor-selective agonists. Stimulation of adenosine receptor subtypes with selective agonists in Ca^{2+} media produced a dose-dependent increase in $[Ca^{2+}]_{cyt}$ with $A_1 > A_{2A} = A_3$, cAMP with A_{2A}, glycogenolysis with $A_1 > A_{2A} > A_3$, and gluconeogenesis with $A_{2A} > A_1 > A_3$, in addition, a decrease in cAMP was observed with $A_1 = A_3$. Comparatively, in Ca^{2+}-free media or with a cell membrane-permeant Ca^{2+} chelator, activation of these adenosine receptors with the same selective agonists produced a smaller and transient rise in $[Ca^{2+}]_{cyt}$ with $A_1 = A_3 > A_2$, no rise in glycogenolysis and gluconeogenesis with $A_3 > A_1$, but a full rise with A_{2A}. Thus, in isolated rat hepatocytes activation of the adenosine A_1 receptor triggered Ca^{2+}-mediated glycogenolysis, activation of the adenosine A_{2A} receptor stimulated cAMP-mediated gluconeogenesis, and activation of the adenosine A_3 receptor increased $[Ca^{2+}]_{cyt}$ and decreased cAMP with minor changes in glycogen metabolism. © 2002 Published by Elsevier Science B.V.

Keywords: Adenosine receptor; Adenosine agonist; Ca^{2+}; cAMP; Glycogenolysis; Gluconeogenesis

1. Introduction

Adenosine displays diverse biological effects through cell-surface receptors in a wide variety of tissues. Four adenosine receptor subtypes, namely A_1, A_{2A}, A_{2B} and A_3, have been identified by convergent data from molecular, biochemical, and pharmacologic studies (Ralevic and Burnstock, 1998). The most widely recognized transduction signals for adenosine A_1 and A_3 receptors are a decrease in cAMP and an increase in Ca^{2+} mobilization, and for the adenosine A_{2A} receptor an increase in cAMP (Ralevic and Burnstock, 1998). However, each adenosine receptor mediates a broad range of signaling responses that may be caused by its coupling to different G proteins (Linden, 1991; Olah and Stiles, 1995; Palmer et al., 1995), although coupling via cytosolic factors has been reported for the adenosine A_1 receptor (Kirsh et al., 1990). In addition, coupling to different signaling pathways is suggested in striatal nerve terminals: one is linked to G_s with subsequent activation of adenylate cyclase and protein kinase A, and another is a cholera toxin-insensitive G protein with the ability to stimulate protein kinase C (Gubitz et al., 1996). Complexity is not limited to signal transducing mechanisms, as opposite biological effects might be caused by micromolar vs. nanomolar concentrations of an adenosine A_3 receptor agonist (Yao et al., 1997). Furthermore, activation of adenosine A_1 and A_3 receptors is required to obtain a single maximal response (Von Lubitz et al., 1994). Therefore, results from one system to another might lead to invalid conclusions.

In liver cells, adenosine and some of its analogs modify the cAMP pool and increase glycogenolysis, gluconeogenesis, and Ca^{2+} mobilization. Hence, identification of the specific adenosine receptor subtype involved in hepatic glycogenolysis and gluconeogenesis and the role of cAMP and Ca^{2+} linking the stimulated adenosine receptor with the biochemical response is desirable. To date, it is known that mRNA encoding the four adenosine receptors has been

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detected in moderate (A_3) or in less than moderate amounts (A_1, A_{2A}, and A_{2B}) in rat liver (Stehle et al., 1992; Salvatore et al., 1993; Dixon et al., 1996), i.e., stimulation of one or more of these four adenosine receptors might start the metabolic response in liver. Information about this may be of particular interest to those who work with adenosine in its therapeutic context.

One approach to gain insight into the question posed is to use selective agonists for each adenosine receptor subtype, in which a high selectivity of agonists based on binding assays translates into a high selectivity in functional assays. For practical purposes in this work, inclusion of adenosine agonists as selective or non-selective was based on data reported in the review of Ravelic and Burnstock (1998). Old data on the hepatic actions of the natural adenosine receptor agonist on glycogenolysis, gluconeogenesis, cAMP and Ca^{2+} are not included in this paper because they do not contribute to solving the problem depicted. Data concerning the effects of selective and non-selective adenosine A_1, A_{2A}, and A_3 receptors on glycogenolysis, gluconeogenesis, cAMP, and Ca^{2+} are summarized below.

Increased hepatic glycogenolysis was obtained with N^6-cyclohexyladenosine (CHA) and (−)-N^6-(2-phenylisopropyl)adenosine (R-PIA) (Buxton et al., 1987), which are considered adenosine A_1 receptor-selective agonists. Additionally, activation of glycogenolysis was obtained with a broad group of non-selective adenosine receptor agonists: 2-chloroadenosine (CADO) (Bartrons et al., 1984), CADO and 5-chloro-5′-deoxyadenosine (CDADO) (Hoffer and Lowenstein, 1986), 5′-*N*-ethylcarboxamidoadenosine (NECA), R-PIA and CADO (Buxton et al., 1987), and NECA and an unidentified isomer of N^6-phenylisopropyladenosine (PIA) (Stanley et al., 1987). Therefore, specific activation of adenosine A_{2A} or A_3 receptors to promote glycogenolysis is not evident. Hepatic gluconeogenesis was stimulated with CADO (Bartrons et al., 1984) and NECA (Zentella de Piña et al., 1989), two non-selective adenosine receptor agonists; thus, the adenosine receptor responsible for this increase in gluconeogenesis has not been identified. A recent report by Harada et al. (2001) suggests that adenosine receptor agonist-induced glucose production in primary cultured rat hepatocytes is mediated through the adenosine A_{2B} receptor.

A decreased pool of cAMP in hepatocytes was reported with R-PIA (Nagy and DeSilva, 1994; Robles-Flores et al., 1995) and N^6-cyclopenthyladenosine (CPA) (Robles-Flores et al., 1995), both selective agonists for the adenosine A_1 receptor. Activation of adenylate cyclase from liver membranes was reported with NECA and PIA (Londos et al., 1980) and an increase in hepatic cAMP was found with CADO (Bartrons et al., 1984) and NECA (Nagy and DeSilva, 1994; Harada et al., 2001); all these agents are non-selective adenosine receptor agonists. In more complex experiments, the glucagon-mediated increase in hepatic cAMP was impaired by the adenosine A_1 receptor-selective agonists R-PIA (Nagy and DeSilva, 1994) and CPA (Robles-Flores et al., 1995), whereas 2-*p*-(2-carboxyethyl) phenethylamine-5-*N*-ethylcarboxamido-adenosine hydrochloride (CGS-21680), the adenosine A_{2A} receptor-selective agonist, did not inhibit glucagon-mediated cAMP production (Nagy and DeSilva, 1994). In summary, activation of the adenosine A_1 receptor decreases the hepatic pool of cAMP, and the liver has the ability to increase cAMP through activation of an adenosine receptor that is probably of the A_{2A} subtype.

2. Material and methods

2.1. Hepatocytes isolation

Male Wistar rats (150–200 g) were anesthetized with ether and cells were isolated by the collagenase perfusion method (Berry and Friend, 1969) modified by Guinzberg et al. (1987). Cell viability was >90% as determined by the Trypan blue (0.4%) exclusion method. Experiments were performed in duplicate with 30–40 mg wet weight of hepatocytes.

2.2. Gluconeogenesis

Hepatocytes from 24-h starved rats were incubated at 37 °C in an atmosphere of O_2/CO_2 (95%/5%) for 60 min in a gyratory water bath in Krebs–Ringer containing 10 mM lactate and each of the chosen adenosine receptor-selective agonists. At the end of the incubation period, tubes were placed in ice-cold water, spun in a clinical centrifuge, and glucose release was measured in the supernatant by the glucose oxidase method (Fales, 1963).

2.3. Glycogenolysis

Hepatocytes from rats fed ad libitum were incubated as previously mentioned, but without lactate for 45 min in the presence of the adenosine receptor-selective agonists. Glucose was measured as previously indicated.

2.4. Cyclic AMP accumulation

Hepatocytes from rats fed ad libitum were incubated at 37.5 °C for 2 min in the presence of the adenosine receptor-selective agonists. At the end of incubation, 100 μl of 2 N HCl was added to each tube. Tubes were heated in a boiling water bath for 2 min and neutralized by the addition of 100 μl of 2 N NaOH. Cyclic AMP was determined using the Amersham Kit TRK432.

2.5. Quin-2 loading of hepatocytes

The method described by Charest et al. (1983) was followed. Briefly, isolated hepatocytes were diluted in Krebs–Ringer to 40 mg w/w containing 2×10^6 cells/ml and were incubated at 37 °C in an atmosphere of O_2/CO_2

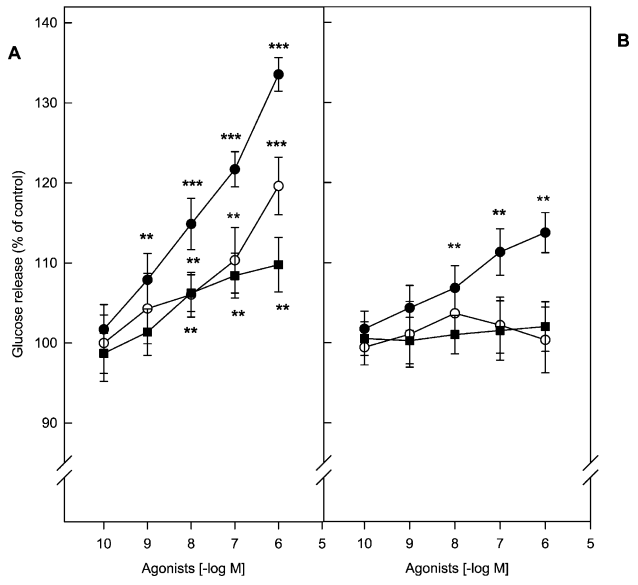

Fig. 1. Effect of adenosine receptor-selective agonists on glycogenolysis. Hepatocytes from rats fed ad libitum were incubated in Krebs–Ringer in the presence (A) or absence (B) of 1.2 mM Ca^{2+} with a different agonist: CCPA (●), CGS-21680 (○), or IB-MECA (■). Results are expressed as percentage of control value: 64.6 ± 4.4 and 49.5 ± 4.3 μmol of glucose formed/45 min per g wet weight, with or without extracellular $[Ca^{2+}]_e$, respectively. Each value represents means ± S.E. of four independent experiments, each performed in duplicate. Statistical significance vs. control is indicated: * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

(95%/5%) for 10 min. Hepatocytes were incubated for 20 additional min in the presence of 100 μM {2-(2-bis-{carboxymethylamino-5-methylphenoxy}methyl-6-methoxy-8bis(carboxymethyl)aminoquinoline tetrakis(acetoxymethylester)} (quin-2-AM), added from 20 mM stock solution in dimethyl sulfoxide (DMSO). After incubation with quin-2-AM, cells were washed twice by centrifugation at 500 rpm/3 min in a clinical centrifuge. Liver cells were distributed in 200-μl aliquots in Eppendorf microfuge tubes and immersed in ice to be used within the following 5 min. $[Ca^{2+}]_{cyt}$ was evaluated in these cells using $K_d = 115$ nM and was calculated as described by Tsien et al. (1982).

2.6. Adenosine receptor-selective agonists used

2-Chloro-N^6-cyclopentyladenosine (CCPA) with a K_d value of 0.4 nM for the adenosine A_1 receptor and a K_d value of 3900 nM for the adenosine A_{2A} receptor (Lohse et al., 1988) was used to stimulate the adenosine A_1 receptor. For adenosine A_{2A} receptor stimulation, CGS-21680 was used, which has a K_d of 15 nM for this receptor and a K_d of 2,600 nM for the adenosine A_1 receptor (Jarvis et al., 1989). For the adenosine A_3 receptor, N^6-(3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine (IB-MECA) was used with a K_d for this receptor of 1.1 nM (Gallo Rodríguez et al., 1994). These adenosine receptor-selective analogs were purchased from RBI (Natick, MA, USA).

2.7. Other reagents and statistical analysis

The permeant, highly selective Ca^{2+} chelating agent 1,2,-bis-(-2-aminophenoxi) ethane *N*,*N*,*N*,*N*′ -tetraacetic acetomethoxy ester (BAPTA-AM) was used at 10 μM. Under our experimental conditions, this agent did not affect cell viability. All other reagents were obtained from Sigma (St. Louis, MO, USA). Results were compared by the use of Student's *t*-test.

3. Results

3.1. Glycogenolysis

Glucose release was promoted in a dose-dependent fashion by the three adenosine receptor-specific agonists used: the potency order of the adenosine receptor type agonists was $A_1 > A_{2A} > A_3$ (Fig. 1A). These data support previous reports that showed that the adenosine A_1 receptor-selective agonists, namely CHA and R-PIA, increased the hepatic glucose release (Buxton et al., 1987). New information indicates a moderate stimulation of glycogenolysis by adenosine A_{2A} and A_3 receptor-selective agonists. Elimination of Ca^{2+} from the incubation mixture led to a significant decrease in the response to the adenosine A_1 receptor-selective agonist and to no response to adenosine A_{2A} and A_3 receptor-selective agonists (Fig. 1B). In this way, $[Ca^{2+}]_{cyt}$ is a triggering event involved in the glycogenolytic response to these selective agonists for adenosine receptors.

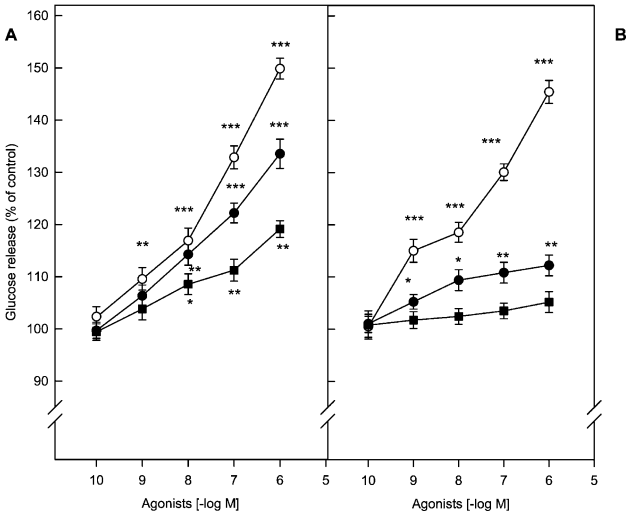

Fig. 2. Effect of adenosine receptor-selective agonists on gluconeogenesis. Hepatocytes from starved rats were incubated in Krebs–Ringer supplemented with 10 mM lactate in the presence (A) or absence (B) of 1.2 mM Ca^{2+} with a different agonist: CCPA (●), CGS-21680 (○), or IB-MECA (■). Results are expressed as percentage of control value, which was 23.8 ± 3.9 and 23.1 ± 1.3 μmol of glucose formed/60 min per g wet weight, with or without $[Ca^{2+}]_e$, respectively. Each value represents means ± S.E. of four independent experiments, each performed in duplicate. Statistical significance vs. control is indicated: * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

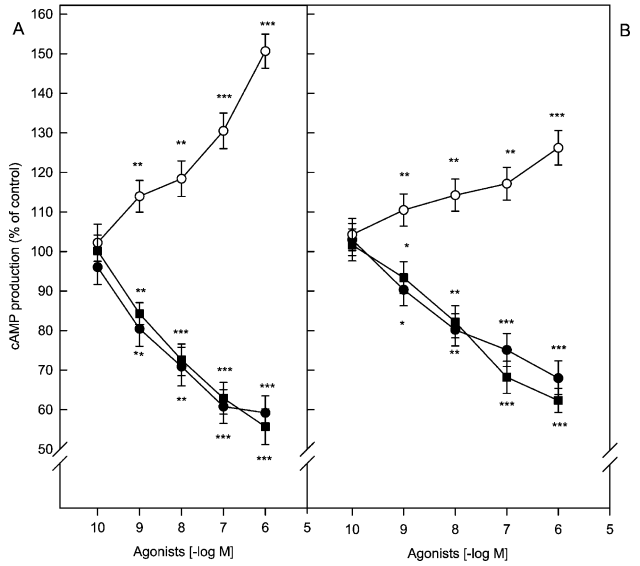

Fig. 3. Effect of adenosine receptor-selective agonists on cAMP production. Hepatocytes from rats fed ad libitum were incubated in Krebs–Ringer in the presence (A) or absence (B) of 1.2 mM Ca^{2+} with a different agonist: CCPA (●), CGS-21680 (○), or IB-MECA (■). Results are expressed as percentage of control value, which was 0.74 ± 0.01 and 0.65 ± 0.07 pmol of cAMP in 2 min/per mg wet weight, with and without $[Ca^{2+}]_e$, respectively. Each value represents means ± S.E. of four independent experiments, each performed in duplicate. Statistical significance vs. control is indicated: * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

3.2. Gluconeogenesis

A dose-dependent increase in the synthesis of glucose from lactate was observed with the selective agonists for adenosine A_1, A_{2A}, and A_3 receptor subtypes used in this study. The adenosine receptor subtype-selective agonist with the best gluconeogenic response was A_{2A}, followed by A_1 and finally by A_3 (Fig. 2A). Once $[Ca^{2+}]_e$ was withdrawn from the incubation media, only the adenosine A_{2A} receptor agonist produced the same stimulation of gluconeogenesis previously recorded in the presence of extracellular Ca^{2+}. In contrast, the adenosine A_3 receptor agonist did not exert a gluconeogenic response and the adenosine A_1 receptor agonist produced a significantly diminished gluconeogenic effect (Fig. 2B).

Table 1
Effect of BAPTA-AM on $[Ca^{2+}]_{cyt}$ in the presence of three adenosine receptor-selective agonists

	None	Agonist		
		A_1	A_{2A}	A_3
Control	115 ± 2.3	227 ± 6.9	201 ± 6.9	193 ± 6.9
		$P<0.001$[a]	$P<0.001$[a]	$P<0.001$[a]
BAPTA-AM	116 ± 1.2	127 ± 11.3	127 ± 10.3	125 ± 6.9
		$P<0.01$[b]	$P<0.01$[b]	$P<0.01$[b]

Isolated hepatocytes (35 mg wet weight) were suspended in 1 ml of Krebs–Ringer with 1.2 mM Ca^{2+} and preincubated with 10 μM BAPTA-AM for 20 min; then, cells were washed twice by centrifugation in Krebs–Ringer with 1.2 mM Ca^{2+} and incubated with quin-2-AM, washed and distributed in 200-μl aliquots as described in Material and methods. Subsequently, cells were treated with each of the selective agonists and cylosolic Ca^{2+} was measured within 3 min. Agonists were used at 1 μM. Each value represents the average ± S.E. of duplicate preparations from four independent experiments. Numbers are in $[Ca^{2+}]_{cyt}$ nM.

[a] Statistical significance, control vs. agonist.

[b] Statistical significance, agonist vs. agonist plus BAPTA-AM.

3.3. Cyclic AMP

The selective adenosine A_{2A} receptor agonist CGS-21680, as a function of the doses used, stimulated cAMP synthesis in isolated rat liver cells (Fig. 3A). Data obtained for the adenosine A_1 receptor-selective agonist showing a

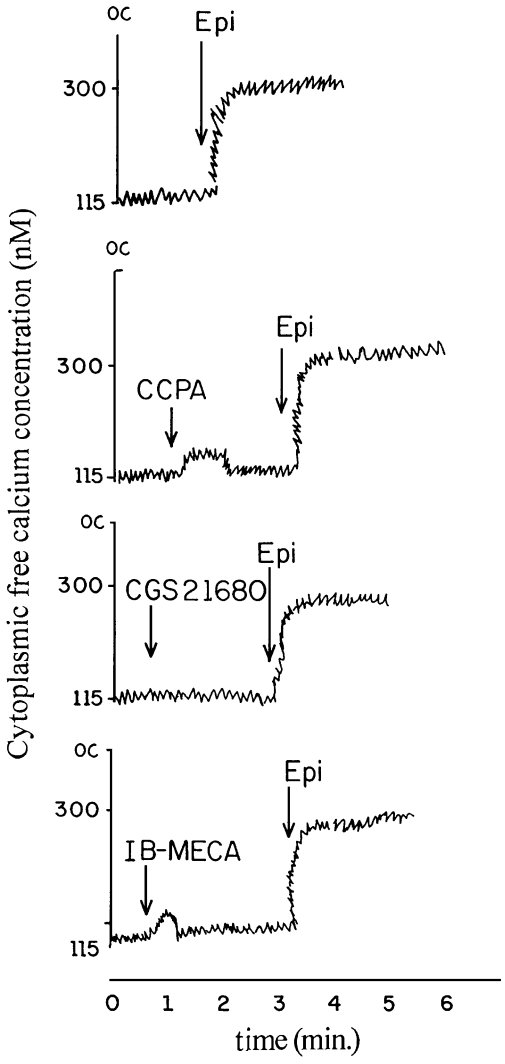

Fig. 4. Effects of adenosine receptor-selective agonists on $[Ca^{2+}]_{cyt}$ in isolated hepatocytes. Quin 2-loaded hepatocytes (25–30 mg wet weight) were incubated in Krebs–Ringer bicarbonate without calcium and with 1.2 mM EGTA. The following additions were made as indicated: Epi, 1 μM epinephrine; A_1, μM CCPA; A_{2A}, 1 μM CGS-21680, and A_3, 1 μM IB-MECA. The experiment is representative of three different assays performed on different days. Essentially, the same results were obtained each day.

decrease in cAMP (Fig. 3A) confirm prior reports (Nagy and DeSilva, 1994; Robles-Flores et al., 1995). Stimulation with the adenosine A_3 receptor-selective agonist decreased cAMP levels in liver cells (Fig. 3A). The inhibitory effect of both adenosine A_1 and A_3 receptor-selective agonists was dose-dependent. It is noteworthy that an adenosine A_{2A} receptor-selective agonist produced a greater increase in cAMP in hepatocytes incubated with 1.2 mM Ca^{2+} (Fig. 3A) than in the absence of Ca^{2+} (Fig. 3B). The addition of adenosineA_1 or A_3 receptor-selective agonists to isolated hepatocytes resulted in an analogous decrease in the basal [cAMP] observed either in the presence or in the absence of Ca^{2+} in the incubation media (Fig. 3A and B).

3.4. Calcium

When the incubation media contained 1.2 mM Ca^{2+}, stimulation with the chosen adenosine A_1, $A_{2A,}$ and A_3 receptor-selective agonists resulted in an increase in $[Ca^{2+}]_{cyt}$ (Guinzberg et al., 1997 and Table 1). In experiments in which Ca^{2+} was missing from the incubation mixture, changes in $[Ca^{2+}]_{cyt}$ were smaller, transient for adenosine A_1 and A_3 receptor-selective agonists, and absent for the adenosine A_{2A} receptor agonist (Fig. 4). All of these information on the modification of $[Ca^{2+}]_{cyt}$ after treatment with these adenosine receptor-selective agonists is compatible with the idea that the increase in $[Ca^{2+}]_{cyt}$ induced by the adenosine A_{2A} receptor-specific agonist depends on $[Ca^{2+}]_e$, whereas intracellular reservoirs contribute to the increase in $[Ca^{2+}]_{cyt}$ induced by adenosine A_1 and A_3 receptor agonists.

The physiologic role of $[Ca^{2+}]_{cyt}$ in the glycogenolytic and gluconeogenic response to the three adenosine receptor-selective agonists was further demonstrated in the following experiment: liver cells prepared in complete Krebs–Ringer media were pretreated with a Ca^{2+}-permeant chelator such as BAPTA-AM, and glycogenolysis and gluconeogenesis were measured after challenging the cells with each of the selective agonists for adenosine receptors. BAPTA-AM-treated cells showed a small increase in $[Ca^{2+}]_{cyt}$ after activation with the three agonists (Table 1). The stimulation of glycogenolysis and gluconeogenesis observed with 1 μM adenosine A_1 and A_3 receptor-selective agonists was completely abolished in cells pretreated with BAPTA-AM (Table 2), whereas the increase in glycogenolysis and gluconeogenesis was unmodified by the permeant chelator after activation with 1 μM adenosine A_{2A} receptor-selective agonist (Table 2).

Table 2
Effect of BAPTA-AM on gluconeogenesis and glycogenolysis in the presence of three adenosine receptor-selective agonists

Agonist	Glycogenolysis		Gluconeogenesis	
	Without BAPTA-AM	With BAPTA-AM	Without BAPTA-AM	With BAPTA-AM
A_1	135.3 ± 3.5	102.4 ± 2.5[a]	127.9 ± 1.5	98.3 ± 3.4[a]
A_{2A}	120.8 ± 2.1	123.0 ± 1.8	151.1 ± 5.3	149.3 ± 2.6
A_3	111.4 ± 3.1	103.1 ± 2.8[a]	114.5 ± 4.5	100.3 ± 2.5[a]

Experimental conditions are the same as in Table 1, except that cells were not washed after treatment with BAPTA-AM, nor were they incubated with quin-2-AM. Results are expressed as percentage of control values: gluconeogenesis (in μmol of glucose formed/60 min per g wet weight) without BAPTA-AM 23.8 ± 3.9, with BAPTA-AM 21.5 ± 4.6, and glycogenolysis (in μmol of glucose formed/45 min per g wet weight) without BAPTA-AM 64.6 ± 4.4, with BAPTA-AM 47.9 ± 3.5. Each value represents the average ± S.E. of duplicate samples from four independent experiments.

[a] Statistical significance, agonist vs. agonist plus BAPTA-AM, $P<0.01$.

4. Discussion

The most extensively studied adenosine receptor subtype in hepatic cells is the A_1 type (Ravelic and Burnstock, 1998). In liver, two adenosine A_1 receptor-specific agonists, CHA and R-PIA, increased glycogenolysis (Buxton et al., 1987); R-PIA and another adenosine A_1 receptor-specific agonist, CPA, decreased cAMP levels (Nagy and DeSilva, 1994; Robles-Flores et al., 1995), while CCPA, a fourth adenosine A_1 receptor-specific agonist, elevated $[Ca^{2+}]_{cyt}$ and doubled the rate of ureagenesis (Guinzberg et al., 1997). In a different system, a single species of adenosine A_1 receptor expressed in Chinese hamster ovary (CHO) cells not only inhibited cAMP accumulation but also stimulated phospholipase C (Akbar et al., 1994). Data from this study confirm previous findings and provide new information on the adenosine A_1 receptor subtype in the liver. Treatment of hepatic cells with CCPA stimulated glycogenolysis (Fig. 1), gluconeogenesis (Fig. 2), and $[Ca^{2+}]_{cyt}$ (Table 1) and resulted in a decrease in cAMP levels (Fig. 3). Among the three adenosine receptor-specific agonists studied, A_1 was the most efficacious in producing a glycogenolytic response (Fig. 1). In addition, the adenosine A_1 receptor-specific agonist stimulated gluconeogenesis to a lesser extent (Fig. 2). Both responses were strictly dependent on the presence of $[Ca^{2+}]_{cyt}$ in that both were absent when free Ca^{2+} was chelated by BAPTA-AM (Table 2). An unsolved question is the origin of the Ca^{2+} that led to an increase in $[Ca^{2+}]_{cyt}$ and thus to stimulate glycogenolysis and gluconeogenesis. A large increase in glycogenolysis and gluconeogenesis was observed when $[Ca^{2+}]_{cyt}$ doubled, mainly due to the contribution of $[Ca^{2+}]_e$ (Table 1), but a moderate increase in glycogenolytic and gluconeogenic response was recorded (Figs. 1 and 2) when Ca^{2+} was supplied exclusively from cell organelles (Fig. 4). In a previous report from our research group, addition of increasing concentrations of CCPA mediated a 50% increment in the rate of ureagenesis in the absence of $[Ca^{2+}]_e$ (Guinzberg et al., 1997).

In *Xenopus* oocytes, Y1 adrenal cells, and dog thyrocytes, transfection with DNA for the adenosine A_{2A} receptor subtype resulted in activation of an adenylate cyclase with an increase in the cAMP pool (Maenhaut et al., 1990). In

hepatic cells, stimulation of the adenosine A_{2A} receptor with CGS-21680 resulted in a dose-dependent increase in cAMP (Fig. 3). Moreover, cAMP-independent signaling has been suggested for adenosine A_{2A} receptors on nerve terminals (Kirk and Richardson, 1995; Gubitz et al., 1996) and in neutrophils (Revan et al., 1996). In the liver, the adenosine A_{2A} receptor-selective agonist accelerated glycogenolysis (Fig. 1) and elicited the greatest stimulation of gluconeogenesis obtained with the adenosine receptor-selective agonists used in this study (Fig. 2). In addition, this agonist increased $[Ca^{2+}]_{cyt}$ at the expense of the incubation media only (Table 1), but not at the expense of the organelle pools (Fig. 4). In previous experiments in which hepatocytes were stimulated with the same adenosine A_{2A} receptor-selective agonist there was an increase in $[Ca^{2+}]_{cyt}$ and the rate of urea synthesis (Guinzberg et al., 1997). The relationship between stimulation of adenosine A_{2A} receptor subtype and Ca^{2+} is complex: $[Ca^{2+}]_e$ was required to obtain the full increase in cAMP promoted by stimulation with the selective agonist (Fig. 3A and B), but the permeant chelator BAPTA-AM did not modify the acceleration of glycogenolisis or gluconeogenesis observed with the adenosine A_{2A} receptor-specific agonist (Table 2); additionally, in cells incubated in a Ca^{2+}-free medium this agonist was incapable of increasing $[Ca^{2+}]_{cyt}$ from cellular organelles (Fig. 4).

The suggestion made by Harada et al. (2001) that hepatic glucose production is induced via adenosine A_{2B} receptor stimulation deserves some comment. Their suggestion was based on two grounds. First, the order of potency to stimulate glucose production in a dose-dependent manner was NECA>CPA>CGS21680, "...consistent with the previously reported order of response toward the A_{2B} receptor" (Harada et al., 2001). It is difficult to follow the reasoning that the order of potency to stimulate hepatic cells with NECA, a non-selective adenosine agonist, CPA, an adenosine A_1 receptor-specific agonist, and CGS 21680, an adenosine A_{2A} receptor-specific agonist, leads to the conclusion that the stimulated adenosine receptor belongs to the A_{2B} subtype. Second, the following three adenosine receptor-specific antagonists, (*E*)-(2*R*)-1-[3-phenylpyrazolo[1,5-*a*]pyridin-3-yl)acryloyl]-2-piperidineethanol for the adenosine A_1 receptor, (*E*)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine for the adenosine A_{2A} receptor, and 6-carboxymethyl-5,9-dihydro-9-methyl-2-phenyl-[1,2,4]-triazolo [5,1-*a*][2,7] naphthyridine for the adenosine A_3 receptor, showed only marginal effects on NECA-induced glucose production in rat hepatocytes. Contrary to the conclusion of Harada et al. (2001), it might be argued that in the latter experiment the adenosine receptor-selective antagonist used impaired the NECA stimulation of one adenosine receptor subtype, but allowed the non-selective NECA, stimulation of the remaining non-inhibited adenosine receptor subtypes, not only the A_{2B} receptors.

Previous studies with liver cells stimulated an adenosine A_3 receptor-specific agonist were performed with N^6-2-(4-aminophenyl)ethyladenosine (APNEA). These experiments showed calcium-dependent stimulation of the rate of urea synthesis and an increase in $[Ca^{2+}]_{cyt}$ from $[Ca^{2+}]_e$ (Guinzberg et al., 1997). Experiments with transfected CHO cells demonstrated that the adenosine A_3 receptor subtype is coupled to both $G_{i\alpha\text{-}2}$, $G_{i\alpha\text{-}3}$ proteins and, to a lesser extent, to $G_{q+11\alpha}$-subunit proteins (Palmer et al., 1995). In rat basophilic leukemia cells, adenosine A_3 receptor stimulation activated phospholipase C and elevated intracellular Ca^{2+} (Ali et al., 1990). Stimulation of adenosine A_3 receptors from rat striata transfected into CHO cells inhibited forskolin-stimulated adenylate cyclase (Zhou et al., 1992). In liver cells, the adenosine A_3 receptor-selective agonist used here, IB-MECA, decreased cAMP levels (Fig. 3) and increased $[Ca^{2+}]_{cyt}$, preferably from the extracellular medium rather than from the organelle pools (Table 1, Fig. 4). In addition to these effects, it appears unlikely that there is a physiological relationship between adenosine A_3 receptor activation and hepatic glucose metabolism.

Experiments are in progress to gain insight into the relationship between each adenosine subtype stimulation and the interplay among $[Ca^{2+}]_e$, $[Ca^{2+}]_{cyt}$, and Ca^{2+} from cell organelles.

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