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Functional characterization of the adenosine receptor contributing to glycogenolysis and gluconeogenesis in rat hepatocytes

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Abstract

The adenosine receptor subtype mediating glucose production by glycogenolysis and gluconeogenesis was studied in primary cultured rat hepatocytes. Adenosine and adenosine agonists caused cyclic AMP accumulation in rat hepatocytes. The order of potency was 5′-*N*-ethylcarboxamidoadenosine (NECA)>*R*(−)-N^6-(2-phenylisopropyl)adenosine (RPIA)>adenosine>2-[*p*-(carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680). Furthermore, adenosine agonists stimulated glycogenolysis and gluconeogenesis. The order of potency was NECA>RPIA>CGS21680. The rank order of potency is typical for adenosine A_{2B} receptors. Glycogenolysis stimulated by NECA was fully inhibited by nonselective adenosine antagonists, 9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine (CGS15943). However, the adenosine A_{2A} receptor-selective antagonist, 8-(3-chlorostyryl)caffeine (CSC), and the adenosine A_1 receptor-selective antagonist, (+)-(*R*)-[(*E*)-3-(2-phenylpyrazolo[1,5-alpha]pyridin-3-yl)acryloyl]-2-piperidine ethanol (FK453), had a low inhibitory potency. A strong correlation was found between the inhibitory effect of adenosine antagonists on NECA-induced glucose production and that on intracellular cyclic AMP generation in rat hepatocytes. Our results suggest that adenosine stimulates cyclic AMP formation and regulates glycogenolysis and gluconeogenesis, most likely through the adenosine A_{2B} receptor subtype in rat hepatocytes.

Keywords: Adenosine; A_{2B} receptor; Glycogenolysis; Gluconeogenesis; Hepatocyte, rat; cAMP

1. Introduction

Adenosine has been demonstrated to regulate many physiological actions through extracellular surface G-protein-coupled receptors. Adenosine receptors are classified into four subtypes (A_1, A_{2A}, A_{2B}, A_3) and are involved in the regulation of adenylate cyclase. Adenosine A_1 and A_3 receptors inhibit cyclic AMP production via G_i, whereas adenosine A_{2A} and A_{2B} receptors stimulate it via G_s (Collis and Hourani, 1993; Pierce et al., 1992). Recently, the availability of adenosine receptor-selective agonists and antagonists has facilitated the classification of adenosine receptors on the basis of the rank order of potency of receptor agonists and antagonists in receptor binding and functional assays. The adenosine A_1 receptor was differentiated from the adenosine A_2 receptor by the adenosine A_1 receptor-selective agonists, N^6-cyclopentyladenosine (CPA) and *R*(−)-N^6-(2-phenylisopropyl)adenosine (RPIA) (Lohse et al., 1988). The adenosine A_2 receptor has been further divided into A_{2A} and A_{2B} subtypes, high-affinity and low-affinity receptors, respectively. These receptors differ by approximately 100-fold in affinity for adenosine (Daly et al., 1983). Although a selective ligand for adenosine A_{2B} receptor has not yet been developed, an adenosine A_{2A} receptor-selective agonist, 2-[*p*-(carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680), is available. Generally, 5′-*N*-ethylcarboxamidoadenosine (NECA), which is a nonselective adenosine agonist, and CGS21680, which is an adenosine A_{2A} receptor-selective agonist, have been widely used as tools to discriminate the effects mediated by the two G_s-coupled receptors (Lupica et al., 1990; Jarvis et al., 1989). For example, in human platelets, which predominantly

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contain adenosine A_{2A} receptors, CGS21680 and NECA showed similar potency in stimulating cyclic AMP generation (Feoktistov and Biaggioni, 1993). In contrast, in adenosine A_{2B} receptor-expressing cells, CGS21680 was ineffective, while NECA was a potent agonist (Alexander et al., 1996; Cooper et al., 1997; Feoktistov and Biaggioni, 1993, 1997; Feoktistov et al., 1994).

Furthermore, adenosine A_1 receptor-selective antagonists, (+)-(*R*)-[(*E*)-3-(2-phenylpyrazolo[1,5-alpha]pyridin-3-yl)acryloyl]-2-piperidine ethanol (FK453) (Terai et al., 1995) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Lohse et al., 1987), an adenosine A_{2A} receptor-selective antagonist, 8-(3-chlorostyryl)caffeine (CSC) (Jacobson et al., 1993), and a nonselective adenosine receptor antagonist, 9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine (CGS15943) (Kim et al., 1998), are useful tools to discriminate between effects mediated by adenosine A_1, A_{2A} or A_{2B} receptors.

It has been reported that adenosine stimulates glycogenolysis via activation of glycogen phosphorylase in isolated rat hepatocytes (Hoffer and Lowenstein, 1986; Oetjen et al., 1990), and stimulation of glycogenolysis by adenosine is mediated through the A_2 receptor in perfused rat liver (Buxton et al., 1987). Furthermore, 2-chloroadenosine, a nondegradable adenosine A_2 receptor agonist, has been shown to stimulate gluconeogenesis by about 20–25% in isolated rat hepatocytes (Bartrons et al., 1984). The gluconeogenic action of adenosine in rat hepatocytes was reported to be mediated by its interaction with receptors that activate the adenylate cyclase system (Pina et al., 1989). These studies suggest that the stimulation of glycogenolysis and gluconeogenesis by adenosine is mediated through the adenosine A_2 receptor in rat hepatocytes. However, it is not known which receptor subtype, A_{2A} or A_{2B}, plays the predominant role. In this study, we tried to identify the receptor subtype primarily associated with these aspects of glucose metabolism in rat hepatocytes by using various metabolically stable adenosine agonists and antagonists.

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 180–280 g (Charles River Japan, Tokyo, Japan), were housed in our laboratories according to the Guidelines for Care and Use of Laboratory Animals of our company. The animal room was maintained at 23 ± 1 °C and 45 ± 5% relative humidity with a 12-h light/dark cycle. Animals were given MF (Oriental Yeast, Tokyo, Japan) as solid food and tap water was given ad libitum.

2.2. Rat hepatocyte culture

Hepatocytes were isolated by a modification of the method previously described (Molde'us et al., 1978). Rats were anesthetized with sodium pentobarbital and the liver was perfused via the portal vein with liver perfusion medium (Gibco BRL, New York, USA) for 10 min, followed by liver digest medium (Gibco BRL) for 10 min at a flow rate of 30 ml min^{-1}. Hepatocytes were suspended by gentle disruption of the digested liver in Williams' E medium containing 10% fetal bovine serum, 0.1 μM insulin, 1 μM dexamethasone, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were plated at 6×10^5 cells/well in collagen-coated, six-well plates or 1.5×10^5 cells/well in collagen-coated, 24-well plates at 37 °C. After incubation for 2 h, nonadherent cells were removed by aspiration, the medium was replaced with fresh Williams' E medium, and culture was continued for 24 h. These hepatocytes were used for the following experiments.

2.3. Cyclic AMP assay with rat hepatocytes

Hepatocytes in 24-well plates were preincubated in fresh Krebs–Ringer Buffer (KRB) containing 10 mM HEPES for 30 min at 37 °C, and adenosine agonists of various concentrations in the presence or absence of selected antagonists were added to the appropriate wells contaning a phosphodiesterase inhibitor, Ro-20-1724, and 1 U ml^{-1} adenosine deaminase. In the case of adenosine itself, adenosine deaminase was not used. After 15 min, supernatants were aspirated and 0.1 N HCl was added to terminate the reaction. Cyclic AMP concentration was determined using a commercially available enzyme immunoassay. Protein concentration was determined using Bio-Rad protein assay reagent.

2.4. Glucose production in rat hepatocytes

Rat hepatocytes in 24-well plates were preincubated in KRB gassed with 95% O_2/5% CO_2 for 30 min at 37 °C, and adenosine agonists or antagonists were simultaneously added to the appropriate wells in the presence of 1 U ml^{-1} adenosine deaminase. After further incubation for 1 h, glucose released into the medium from the hepatocytes was determined with a Glucose test Wako, a kit using the glucose oxidase method.

2.5. Glycogen content in primary cultured rat hepatocytes

Adenosine agonists or antagonists in the presence of 1 U ml^{-1} adenosine deaminase were simultaneously added to rat hepatocytes cultured in six-well plates. After a 1-h incubation, supernatants were aspirated and the hepatocytes attached to the wells were digested in 2 ml of 30% KOH for 10 min. Then, the digest was cooled to room temperature, and 0.8 ml of 2% Na_2SO_4 and 4 ml of absolute ethanol were added to it. These tubes were kept at −20 °C overnight to precipitate glycogen and centrifuged at 1000 × *g*, for 10 min. The supernatants were discarded and the precipitate was washed with 2 ml of 66% ethanol. Then, the pellets were dried and dissolved in 0.2 M acetate. Glycogen was digested

into glucose by glucoamylase (10 U ml^{-1}) for 60 min at 37 °C, and produced glucose levels were measured by the enzymatic method described previously. The glycogen content is expressed as micrograms of glucose per well (Reddi and Jyothirmayi, 1992).

2.6. Gluconeogenesis from [^{14}C]lactate in rat hepatocytes

Isolated hepatocytes cultured in 24-well plates for 24 h were washed twice with 1 ml of KRB solution. Hepatocytes were stimulated with increasing concentrations of an adenosine agonist, NECA, RPIA and CGS21680, followed by addition of 0.2 μCi of [^{14}C]lactate. The final concentration of lactate, including [^{14}C]lactate, was 5 mM (Ciaraldi et al., 1990). After a 4-h incubation, [^{14}C]glucose generated from [^{14}C]lactate was separated on an anion exchange column (AG-1-x8) and its radioactivity was measured with a scintillation counter (Packard Instrument, Meriden, CT, USA).

2.7. Cyclic AMP assay in HepG2 cells

HepG2 cells were grown in Dulbecco's modified Eagles' medium (DMEM) containing 10% fetal bovine serum. Hep G2 cells in 24-well plates were preincubated in fresh Krebs–Ringer Buffer KRB containing 10 mM HEPES for 30 min at 37 °C, and adenosine agonists at various concentrations were added to the appropriate wells containing a phosphodiesterase inhibitor, Ro-20-1724, and 1 U ml^{-1} adenosine deaminase. After 15 min, supernatants were aspirated and 0.1 N HCl was added to terminate the reaction. Cyclic AMP concentration was determined using a commercially available enzyme immunoassay.

2.8. Data analysis

Statistical analysis was conducted with the software package SAS 6.12 (SAS Institute Japan, Tokyo, Japan). The IC_{50} and EC_{50} values were calculated with a nonlinear regression method.

2.9. Chemicals and drugs

The following chemicals and drugs were used: sodium pentobarbital (Dainippon Pharmaceutical, Osaka, Japan); adenosine, NECA, DPCPX, RPIA, CGS15943, dexamethasone and insulin (Sigma, St. Louis, MO, USA); FK453 was synthesized in the Eisai (Akahane et al., 1996); adenosine deaminase (Boehringer Mannheim, Indianapolis, IN, USA); liver perfusion medium, liver digest medium, Williams' E and antibiotics (penicillin and streptomycin) (Gibco BRL); Glucose test Wako and glucoamylase (Wako, Tokyo, Japan); CSC, Ro-20-1724 and CGS21680 (RBI, Natick, CA, USA); anion exchange column (AG-1-x8) (Bio-Rad, California, USA); [^{14}C]lactate (specific radioactivity, 174.6 mCi $mmol^{-1}$) and cyclic AMP EIA system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

3. Results

3.1. Effects of adenosine, NECA, RPIA and CGS21680 on cyclic AMP generation in rat hepatocytes

Stimulatory effects of adenosine and the adenosine agonists, NECA, RPIA and CGS21680, on cyclic AMP generation were investigated in rat hepatocytes (Fig. 1). Basal accumulation of cyclic AMP was 5.57 ± 0.47 (pmol/mg protein). NECA stimulated cyclic AMP generation in a dose-dependent manner in hepatocytes. In this experiment, when the cyclic AMP level in response to 100 μM NECA was chosen as the maximal response for calculating EC_{50} values, the EC_{50} of NECA calculated from the maximum response was 0.58 ± 0.18 μM, whereas 100 μM adenosine, 100 μM RPIA or 100 μM CGS21680 did not elicit this maximum level. The cyclic AMP levels in response to 100 μM adenosine, 100 μM RPIA and 100 μM CGS21680 were only 24.7%, 43.9% and 17.0% of that produced by 100 μM NECA, respectively. Therefore, the rank order of potency was NECA>RPIA>adenosine>CGS21680.

3.2. Glycogenolytic effects of NECA, RPIA and CGS21680 in rat hepatocytes

Stimulation of glycogenolysis by adenosine agonists was investigated by measuring glucose production in the medium and glycogen levels in the hepatocytes (Fig. 2A and B). NECA, RPIA and CGS21680 stimulated glucose production in a concentration-dependent manner. Maximum response to NECA, RPIA and CGS21680 was attained at 10^{-4} M and the EC_{50}s calculated from the maximum response to these agonists were 0.12 ± 0.04, 0.77 ± 0.03 and 20.12 ± 5.48 μM, respectively. From these results, the rank order of

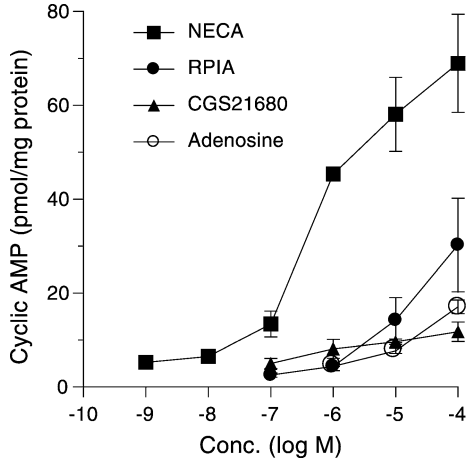

Fig. 1. Adenosine and adenosine receptor agonist-stimulated cyclic AMP production in primary cultured rat hepatocytes. Hepatocytes were incubated with increasing concentrations of NECA (■), RPIA (●), CGS21680 (▲) and adenosine (○) for 15 min in the presence of a phosphodiesterase inhibitor, Ro-20-1724 (100 μM). Cyclic AMP content was determined with an enzyme immunoassay kit from Amersham. Each point represents the mean ± S.E.M. of four experiments in triplicate.

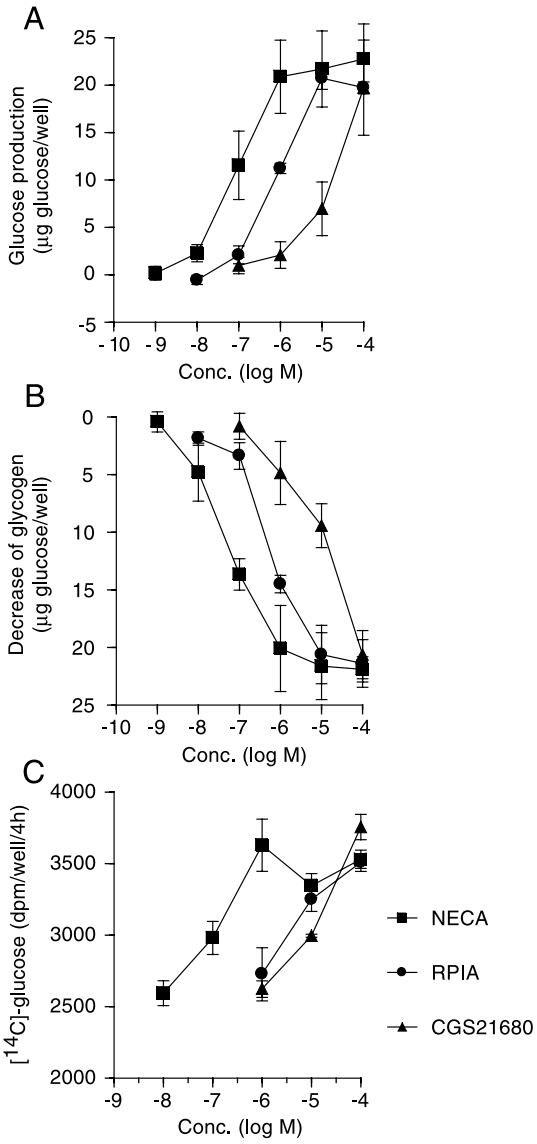

Fig. 2. Adenosine receptor agonist-stimulated glycogenolysis and gluconeogenesis in rat hepatocytes. (A) Effect of NECA (■), RPIA (●) and CGS21680 (▲) on glucose production in rat hepatocytes. (B) Effect of NECA (■), RPIA (●) and CGS21680 (▲) on glycogen level in rat hepatocytes. Hepatocytes were incubated with NECA, RPIA or CGS21680, for 1 h. Glucose released into the medium from the hepatocytes and glycogen content in hepatocytes were measured in the same experiments. (C) Effect of NECA (■), RPIA (●) and CGS21680 (▲) on gluconeogenesis in rat hepatocytes. Incorporation of ^{14}C into [^{14}C]glucose from [^{14}C]lactate stimulated by NECA, RPIA and CGS21680 was monitored for 4 h in rat hepatocytes. [^{14}C]Glucose was separated on an anion exchange column (AG-1-x8) and the radioactivity was measured with a scintillation counter. Each point represents the mean ± S.E.M. of three experiments in triplicate.

potency of these adenosine agonists to stimulate glucose production was NECA>RPIA>CGS21680. Comparison of EC_{50} values showed that the potency of NECA to evoke glucose production was about 168-fold greater than that of CGS21680 (Fig. 2A).

In this experiment, the total amount of glucose released into the medium was simultaneously monitored and was almost identical with the decrease in glycogen content in the hepatocytes. The basal glycogen content was 36.26 ± 3.30 (μg glucose/well). The potency of these adenosine agonists in increasing glucose production was very similar to that in decreasing the glycogen content (Fig. 2B). The EC_{50}s of NECA, RPIA and CGS21680 calculated from the maximum response in terms of glycogen content were 0.08 ± 0.04, 0.60 ± 0.09 and 13.08 ± 1.67 μM, respectively. These results support the idea that glucose production induced by these adenosine agonists is derived from a stimulation of glycogenolysis.

3.3. Effects of NECA, RPIA and CGS21680 on gluconeogenesis in rat hepatocytes

We next examined the direct activation of the gluconeogenic pathway by adenosine agonists by measuring the incorporation of ^{14}C into [^{14}C]glucose from [^{14}C]lactate (Fig. 2C). NECA, RPIA and CGS21680 evoked [^{14}C]glucose production in a concentration-dependent manner in rat hepatocytes. Basal incorporation of [^{14}C] into glucose from [^{14}C]lactate as gluconeogenic activity was 2422.4 ± 16.9 (dpm/well/4h). Maximal response to NECA, RPIA and CGS21680 was attained at 10^{-4} M, and the EC_{50}s of these agonists calculated from the maximum response were 0.14 ± 0.05, 2.74 ± 0.48 and 10.41 ± 1.88 μM, respectively. Thus, the rank order of potency for gluconeogenic activity of adenosine agonists was NECA>RPIA>CGS21680. The rank order of these agonists for gluconeogenesis was similar to that for glycogenolysis and also typical for adenosine A_{2B} receptors.

3.4. Effects of CGS15943, DPCPX, FK453 and CSC on NECA-evoked glucose production in rat hepatocytes

We tried to examine the effect of adenosine antagonists on glucose production via NECA-induced glycogenolysis using an adenosine A_{2A} receptor-selective antagonist, CSC (Jacobson et al., 1993), an adenosine A_1 receptor-selective antagonist, DPCPX and FK453 (Terai et al., 1995), and a nonselective adenosine antagonist, CGS15943 (Kim et al., 1998), in rat hepatocytes (Fig. 3A). The glucose production stimulated by this submaximal concentration of NECA (0.1 μM) was inhibited by CGS15943, DPCPX, FK453 and CSC in a concentration-dependent manner. The IC_{50}s of CGS159 43, DPCPX, FK453 and CSC were 0.28 ± 0.11, 1.03 ± 0.24, 6.59 ± 0.54 and 14.51 ± 3.96 μM, respectively.

3.5. Correlation between the inhibition of NECA-stimulated cyclic AMP generation and glucose production in hepatocytes

We investigated the correlation between the inhibition of NECA-stimulated cyclic AMP generation and glucose production using various adenosine antagonists, i.e., CGS159 43, DPCPX, FK453 and CSC in hepatocytes (Fig. 3B).

Cyclic AMP formation and glucose production in hepatocytes induced by 0.1 μM NECA were inhibited in the presence of selective and nonselective antagonists of adenosine A_1, A_{2A} and A_{2B} receptors. The rank order of pIC_{50} ($-\log(IC_{50})$) values for cyclic AMP formation was CGS15943 (7.83 ± 0.10)>DPCPX (7.49 ± 0.08)>FK453 (6.19 ± 0.06)>CSC (5.91 ± 0.05). The rank order of pIC_{50} values for glucose production was CGS15943 (6.61 ± 0.15)>DPCPX (6.02 ± 0.12)>FK453 (5.18 ± 0.04)>CSC (4.87 ± 0.11).

A strong correlation was found between the inhibitory effect of adenosine antagonists on NECA-stimulated cyclic AMP generation and glucose production in hepatocytes ($r = 0.99$, $P < 0.05$). These results suggest that cyclic AMP is the main second messenger leading to glycogenolysis and gluconeogenesis mediated through the adenosine A_{2B} receptor subtype in rat hepatocytes.

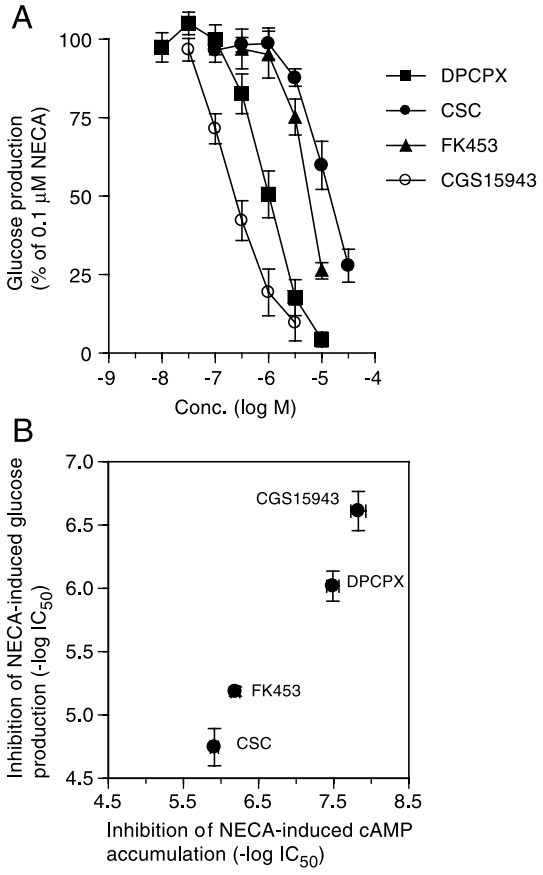

Fig. 3. Effects of adenosine receptor antagonists on NECA-stimulated glucose production in rat hepatocytes. (A) CGS15943 (○), DPCPX (■), FK453 (▲) and CSC (●) and 0.1 μM NECA was simultaneously added to rat hepatocytes and incubated for 1 h. The antagonistic effect is expressed as a percentage of the response induced by 0.1 μM NECA. The values are expressed as a percentage of the response to 100 μM NECA. Each point represents the mean ± S.E.M. of three experiments in triplicate. (B) Correlation between inhibitory effect of adenosine antagonists on 0.1 μM NECA-induced glucose production and that on intracellular cyclic AMP generation in rat hepatocytes ($r = 0.99$, $P < 0.05$). The values are expressed as $-\log(IC_{50})$. Each point represents the mean ± S.E.M. of three experiments in triplicate.

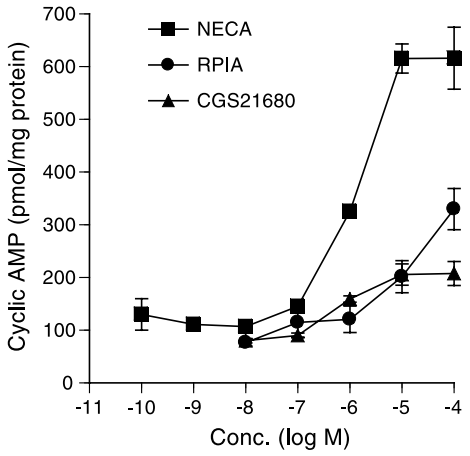

Fig. 4. Adenosine receptor agonist-stimulated cyclic AMP production in HepG2 cells. HepG2 cells were incubated with increasing concentrations of NECA (■), RPIA (●) and CGS21680 (▲) for 15 min in the presence of a phosphodiesterase inhibitor, Ro-20-1724 (100 μM). Cyclic AMP content was determined with an enzyme immunoassay kit from Amersham. Each point represents the mean ± S.E.M. of three experiments in triplicate.

3.6. Effects of NECA, RPIA and CGS21680 on cyclic AMP generation in HepG2 cells

The stimulatory effects of the adenosine agonists, NECA, RPIA and CGS21680, on cyclic AMP generation were investigated in HepG2 cells, human hepatocyte cell lines (Fig. 4). Basal accumulation of cyclic AMP was 56.2 ± 9.90 (pmol/mg protein). NECA stimulated cyclic AMP generation in a dose-dependent manner in HepG2 cells. When the cyclic AMP level in response to 100 μM NECA was chosen as the maximal response for calculating EC_{50} values, the EC_{50} of NECA calculated from the maximum response was 1.25 ± 0.18 μM, whereas 100 μM RPIA or CGS21680 did not elicit such a response. The cyclic AMP levels in response to 100 μM RPIA and 100 μM CGS21680 were only 48.8% and 27.0% of that produced by 100 μM NECA, respectively. Therefore, the rank order of potency was NECA>R-PIA>CGS21680. This rank order is also typical for adenosine A_{2B} receptors.

4. Discussion

The adenosine receptor subtypes mediating glucose production derived from glycogenolysis and gluconeogenesis were studied in primary cultured rat hepatocytes.

There is evidence that the adenosine A_2 receptor is involved in glucose metabolism in rat liver. mRNA for adenosine A_1, A_{2A}, A_{2B} and A_3 receptors has been detected in rat liver by the RT-PCR method (Dixon et al., 1996). Previous findings raise the possibility that the A_2 subtype of adenosine receptor contributes to glycogenolysis and gluconeogenesis in rat hepatocytes. However, it was not clear which subtype of adenosine A_2 receptor, A_{2A} or A_{2B}, played an important role in these processes.

Our results showed that adenosine and adenosine agonists stimulated cyclic AMP formation in a dose-dependent manner, most likely through the adenosine A_{2B} receptor subtype in rat hepatocytes, because the rank order potency was NECA>RPIA>adenosine>CGS21680.

The existence of the adenosine A_{2B} receptor was first established by the isolation of a cDNA, designated RFL9, from rat brain (Stehle et al., 1992). The rank order of potency of adenosine agonists as inducers of cyclic AMP generation in RFL9-transfected cells was NECA>RPIA>CGS21680 (Rivkees and Reppert, 1992).

Previous studies also reported that the same rank order of potency was observed for the human adenosine A_{2B} receptor that was cloned and transfected in Chinese hamster ovary (CHO) cells (Alexander et al., 1996) and an endogenous adenosine A_{2B} receptor expressed in HEK293 cells (Cooper et al., 1997). The lack of activity of the selective adenosine A_{2A} agonist, CGS21680, is an accepted indication of the absence of an adenosine A_{2A} receptor in other experiments (Lupica et al., 1990; Feoktistov and Biaggioni, 1993; Pelletier et al., 2000).

Our results showed that a nonselective adenosine agonist, NECA, evoked glucose production more potently than did RPIA and CGS21680 in primary cultured rat hepatocytes. This result supports that glucose production is stimulated via the adenosine A_{2B} receptor. In this study, the total amount of glucose released from hepatocytes was identical to the decrease in glycogen levels in rat hepatocytes, suggesting that glucose production caused by adenosine agonists was derived from glycogenolysis.

Furthermore, we directly tried to evaluate the effect of adenosine agonists on gluconeogenesis from [^{14}C]lactate as a substrate in rat hepatocytes after a 4-h incubation. Gluconeogenesis from [^{14}C]lactate was evoked in a dose-dependent manner with the rank order of potency of NECA>RPIA>CGS21680.

The rank order of adenosine agonists (NECA>RPIA>CGS21680) involved in glycogeolysis and gluconeogenesis was the same as that observed in previous studies characterizing the adenosine A_{2B} receptor (Alexander et al., 1996; Cooper et al., 1997). These results strongly suggested that adenosine stimulated glycogenolysis and gluconeogenesis via the adenosine A_{2B} receptor in primary cultured rat hepatocytes.

The rank order of potency to inhibit glucose production found in our study was CGS15943>DPCPX>FK453>CSC. The very low inhibitory potency of FK453 and CSC suggested that the adenosine receptor subtype that contributed to glucose production elicited by adenosine agonists was not the A_1 or A_{2A} subtype. DPCPX fully inhibited the glucose production caused by NECA in rat hepatocytes, although DPCPX has been regarded as adenosine A_1 receptor antagonist. However, previous studies reported that DPCPX had a moderate affinity for the adenosine A_{2B} receptor with sufficient potency to inhibit NECA-stimulated cyclic AMP production in human adenosine A_{2B} receptor-expressing HEK293 and CHO.K1 cells (Cooper et al., 1997; Klotz et al., 1998; Pelletier et al., 2000). In addition, it was reported that CGS15943 has a high affinity for adenosine A_1, A_{2A}, A_{2B} and A_3 receptors (Kim et al., 1998). These results also suggest that adenosine-stimulated glucose production occurs via the adenosine A_{2B} receptor, and not the A_1 or A_{2A} subtype.

Previous studies indicate that the intracellular cyclic AMP level is one of the second messengers of the signal transduction pathway leading to glycogenolysis and gluconeogenesis stimulated by adenosine in hepatocytes (Hoffer and Lowenstein, 1986). We tried to examine a correlation between cyclic AMP levels and glucose production caused by 0.1 μM NECA, using various adenosine receptor antagonists (CGS15943, DPCPX, FK453 and CSC) in hepatocytes. Our results showed that there was a strongly positive correlation between inhibitory effect of adenosine antagonists on NECA-induced glucose production and that on intracellular cyclic AMP generation in rat hepatocytes. These data suggested that inhibition of NECA-induced cyclic AMP generation by adenosine antagonists had an inhibitory effect on glucose production through adenosine A_{2B} receptors in rat hepatocytes.

Our data suggested that 100 μM CGS21680 caused glucose production comparable with the NECA-induced maximal response in spite of a relatively low level of cyclic AMP in hepatocytes. One possible explanation is that adenosine agonists contribute to glucose production derived from glycogenolysis and gluconeogenesis via another second messenger besides cyclic AMP in hepatocytes. The most likely candidate is Ca^{2+}, because adenosine increases the intracellular Ca^{2+} level in hepatocytes, and the stimulation of glycogenolysis is suppressed in hepatocytes incubated in Ca^{2+}-free medium (Tinton et al., 1996). Previous studies reported that adenosine increased intracellular Ca^{2+} levels through adenosine A_{2B} receptors in human erythroleukemia cells (Feoktistov et al., 1994). It is likely that the increase in intracellular Ca^{2+} levels through the adenosine A_{2B} receptor subtype in response to adenosine causes glucose production in hepatocytes.

In this study, we did not examine the possibility that adenosine A_3 receptors were present, although it has been reported that the adenosine A_3 receptor is present in rat hepatocytes (Dixon et al., 1996). However, it is unlikely that glucose production elicited by adenosine agonists is mediated through adenosine A_3 receptors because this receptor is known to be resistant to xanthine antagonists such as DPCPX even at concentrations of 10 and 100 μM (Van Galen et al., 1994), and the rank order of potency for rat adenosine A_3 receptor agonists was RPIA = NECA>CGS21680 (Zhou et al., 1992). In the present study, 10 μM DPCPX completely inhibited NECA-stimulated glucose production and the rank order of potency of glucose production in response to adenosine agonists was NECA>RPIA>CGS21680, supporting the idea that the adenosine A_3 receptor subtype is not involved in adenosine-mediated glucose production.

In the present study, we showed that adenosine agonists stimulated cyclic AMP production in HepG2 cells, a human hepatocyte cell line. The results were very similar to those for rat hepatocytes and the rank order was NECA>RPIA>CGS21680. This is the typical for adenosine A_{2B} receptors. These results suggested the possibility that adenosine could regulate glucose metabolism in the human liver.

The role of adenosine in glucose metabolism has been discussed by many researchers. In vivo studies have shown that the administration of adenosine and adenosine agonists elevates blood glucose levels (Bacher et al., 1982; Raberger et al., 1980), suggesting that adenosine stimulates glucose production in the liver or suppresses glucose utilization by peripheral tissues (Challiss et al., 1992). However, there is little clear evidence to show whether adenosine influences either of or both. Our results suggest that the effect of adenosine was, at least in part, due to stimulation of glucose production through glycogenolysis and gluconeogenesis in the rat liver.

We speculate that an adenosine antagonist may block hyperglycemia by inhibition of glucose production and improve insulin resistance in the liver in diabetes. Indeed, we recently reported that adenosine antagonists possessing A_{2B} antagonism decreased blood glucose levels in KKA^{y} mice, an animal model of non-insulin-dependent diabetes mellitus (Harada et al., 2001), although the subtype selectivity of adenosine antagonists on the anti-hyperglycemic effect remains to be investigated in more detail. It is also possible that the adenosine A_{2B} receptor plays an important role in glucose metabolism and insulin action in the liver in type II diabetes.

In conclusion, our results indicate that adenosine stimulated cyclic AMP formation and caused glycogenolysis and gluconeogenesis, most likely via the adenosine A_{2B} receptor subtype in primary cultured rat hepatocytes. Therefore, the possibility arises that adenosine regulates glucose metabolism via the adenosine A_{2B} receptor subtype in the normal physiological state, as well as in pathological states such as ischemia and diabetes.

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