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EFFECTS OF ADENOSINE AND ADENOSINE ANALOGUES ON GLYCOGEN METABOLISM IN ISOLATED RAT HEPATOCYTES*

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Abstract—Adenosine and adenosine analogues were incubated with isolated rat hepatocytes. Adenosine and 5′-deoxy-5′-chloroadenosine stimulated glucose release, glycogen loss, and the conversion of glycogen phosphorylase *b* to *a*. The effect was of short duration for adenosine, but of long duration for 5′-deoxy-5′-chloroadenosine. The effects on glucose release and phosphorylase were blocked by theophylline, an R-receptor blocking agent, but not by nitrobenzylthioinosine or dipyridamol which are nucleoside transport inhibitors. A dose-dependent rise in cyclic AMP concentration was observed in hepatocytes 1 min after adding adenosine. It is concluded that adenosine exerts these effects in liver by activating adenylcyclase. Adenosine may be involved in the short-term regulation of hepatic glycogen phosphorylase.

Adenosine plays an important role in the local regulation of blood flow through heart, brain, and skeletal muscle [1]. A substrate cycle between AMP and adenosine has been proposed as a means by which fine regulation of adenosine levels could be maintained [2]. Recent work by Bontemps *et al.* [3] provides evidence for such a cycle in isolated rat hepatocytes and raises the question of a local regulatory role for adenosine in liver.

Adenosine acts in two different locations to modify the activity of adenylate cyclase. First, it binds to external plasma membrane receptors, termed R receptors, which mediate either an activation (R_a) or an inhibition (R_i) of adenylcyclase activity and corresponding changes in the intracellular concentrations of cyclic AMP. Second, it binds to a site believed to be located on the internal plasma membrane surface, termed the P site, where it inhibits increases in adenylcyclase activity in response to various agonists [4]. R_a receptors have been identified on rat liver plasma membranes [5, 6], and an increase in cyclic AMP concentrations has been observed in rat liver derived clonal cell lines incubated in the presence of 2-chloroadenosine [7]. Until very recently, however, only unphysiologically high concentrations of adenosine have been shown to alter rates of gluconeogenesis, lipogenesis, and fatty acid oxidation [8–11] or to increase cyclic AMP concentrations [11] in liver. In one study, even 200 μM adenosine could not be shown to affect glycogen metabolism or cyclic AMP concentrations in isolated rat hepatocytes [12]. During the course of the work described here, Bartrons *et al.* [13] reported activation of hepatocyte phosphorylase and an increase in cellular cyclic AMP in response to micromolar concentrations of adenosine and the R-receptor agonist 2-chloroadenosine.

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EXPERIMENTAL PROCEDURES

Hepatocyte preparation. Hepatocytes were isolated from livers of 200–400 g male Sprague–Dawley rats obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. Between 10:00 a.m. and noon, fed animals were lightly anesthetized with ether, and the portal vein was exposed rapidly and cannulated. Livers were perfused using a recirculating perfusion apparatus described previously [14]. Hepatocytes were isolated as described by Seglen [15]. The cells were suspended and stored in 10 ml/g wet weight of ice-cold Krebs–Henseleit bicarbonate buffer containing 20 mM glucose and 1% bovine serum albumin, in a 500-ml Erlenmeyer flask gassed with 95% O_2–5% CO_2. The flasks were stoppered, kept in crushed ice, and swirled gently at 20-min intervals. The cells showed over 90% viability by the Trypan blue exclusion method [15] and maintained their viability for up to 5 hr when stored on ice. Experiments were conducted with cells that had been stored for up to 4 hr.

The protein content of hepatocytes was determined by the method of Lowry *et al.* [16] with bovine serum albumin as standard. Hepatocytes from fed rats contained 137 ± 6 mg protein per g wet weight (mean ± SE, N = 11) and 199 ± 5 mg dry weight (N = 6) per g wet weight.

Glucose release and glycogen content of hepatocytes. Hepatocyte suspension (1 ml) was pipetted into a cold 25-ml Erlenmeyer flask and 1.5 ml of ice-cold Krebs–Henseleit bicarbonate buffer containing the test substance but lacking albumin was added.

The flask was gassed with 95% O_2–5% CO_2 and stoppered. At zero-time the flask was placed in a Dubnoff incubator at 37° and shaken 100 times/min. At the desired time, 1 ml of cell suspension containing 40 mg wet weight of cells was removed and pipetted into 1 ml of 1 M perchloric acid and vortexed. The mixture was centrifuged and the supernatant fraction was removed and analyzed for glucose [17]. Glycogen was assayed by a combination of the methods of Lust *et al.* [18] and Keppler and Decker [19]. One milliliter of cell suspension was pipetted into 1 ml of ice-cold 0.12 N HCl and mixed, bringing the pH to approximately 2. Then 0.4 ml of the resulting suspension was added to 0.05 ml of 1 N NaOH in a small screw-cap vial which was closed tightly and placed in a boiling water bath for 60 min. After cooling, 0.05 ml of 1.5 N acetic acid was added to bring the pH to approximately 5. Next, 0.12 ml of a solution of amyloglucosidase in 0.45 M sodium acetate buffer, pH 4.8, was added. The vial was incubated at 37° for 2 hr, 0.15 ml of 1 N perchloric acid was added, and the vial was chilled to precipitate proteins. The mixture was centrifuged, and 0.05 ml of the clear supernatant fraction was assayed for glucose [17].

Phosphorylase assay. The hepatocyte suspensions were diluted and gassed as described above. At times −20 or −10 min, flasks were placed in a water bath at 37° and shaken 100 times/min. At zero time the substance to be tested was added in 0.25 ml Krebs–Henseleit buffer. One minute later and at various subsequent times, 0.2 ml of the suspension was removed for assay.

The phosphorylase assay was conducted in the direction of glycogen breakdown, according to the methods of Thurston *et al.* [20] and Aragón *et al.* [21]. Cell suspension (0.2 ml, containing 8 mg cells) was gently withdrawn with an automatic pipette and frozen instantly by expulsion into a test tube cooled in a dry ice and ethanol bath. Ice-cold homogenization buffer (0.2 ml) was added, and the tubes were kept in crushed ice until the mixture had melted. The homogenization buffer contained 100 mM HEPES* buffer, pH 7.4, 10 mM EDTA, 0.2 M NaF, 10 mM DTT, and 2% Triton X-100. Preliminary experiments showed that vortexing the cells with an equal volume of this buffer gave similar results to the more laborious method of using a Dounce homogenizer to break the cells. Microscopic analysis confirmed that the Triton X-100-treated cells were completely lysed following vigorous vortexing. The assay was begun by adding 0.02 ml of homogenate (0.4 mg cells) to 0.98 ml of assay buffer in a quartz cuvette at 30° to give final concentrations of 50 mM imidazole–HCl, pH 7.0, 7.5 mM potassium phosphate, 15 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DDT, 0.1 mM AMP, 0.58 mM NADP, 5 mg/ml glycogen, 1.75 units/ml glucose-6-phosphate dehydrogenase, and 1.4 units/ml phosphoglucomutase. A Perkin–Elmer double beam spectrophotometer (model 557) was used to measure changes in absorbance at 340 nm minus changes in absorbance at 400 nm. The change in absorbance with time became constant after about 5 min and remained so for at least 20 min. Results are expressed as μmoles of NADPH formed/g wet weight of cells/min. Addition of 0.1 mM AMP to the assay increased the rate of the reaction by 19% over the value obtained in its absence. Further increases in AMP concentration up to 1.7 mM led to no further increase of activity. This is consistent with the findings of Stalmans and Gevers [22], who, working with purified rat liver phosphorylase assayed in the direction of glycogenolysis, found that AMP activates phosphorylase *a* by approximately this amount in the presence of the phosphate concentration used in the assay. Under these conditions, phosphorylase *b* showed no activity even in the presence of AMP. Consistent with their results, we found that hepatocyte homogenates originally showing low phosphorylase activity were not activated by addition of AMP. Thus, the assay system used by us measured only phosphorylase *a*. Because theophylline was used in some experiments, its inhibitory effect on the assay system was tested. Theophylline (0.05 mM) in the assay mixture had no effect, but 0.1, 0.5, and 1 mM theophylline decreased the activity by 10, 17, and 34% respectively. Because samples to be analyzed were diluted 50-fold in the assay mixture, the final concentration of theophylline in the assay mixtures did not exceed 0.05 mM. The glycogen used in the assay was Type VII from the Sigma Chemical Co.; it was purified prior to use by passage through a column of Monobed Amberlite mixed ion exchange resin.

In some experiments, total phosphorylase activity was determined by converting phosphorylase *b* completely to the *a* form using phosphorylase kinase. One milliliter of cell suspension (40 mg cells) was pipetted into a 15-ml Corex tube containing 2 ml of ice-cold Krebs–Henseleit bicarbonate buffer and mixed. The tube was centrifuged at 1500 *g* for 1 min at 0°. The supernatant fraction was removed with a Pasteur pipette, and the pellet was frozen immediately by holding the tube in a dry ice and ethanol bath. The material was stored overnight at −20°. At the time of analysis, 0.36 ml of homogenization buffer was added to give final concentrations of 50 mM HEPES, pH 7.4, 5 mM EDTA, 0.1 M NaF, 5 mM DTT, and 1% Triton X-100. An activating solution was prepared as described by Lederer and Stalmans [23]: it contained 1.5 units/ml of rabbit muscle phosphorylase *b* kinase, 10 mM ATP, 15 mM magnesium acetate, and 0.2 mM cyclic AMP. This was mixed with an equal volume of the cell homogenate and incubated at 37° for 30 min. An aliquot (0.02 ml) was then assayed for phosphorylase activity as described above. The concentration of phosphorylase *b* kinase was chosen to give maximum activation, without adding an appreciable amount of rabbit muscle phosphorylase which was present as an impurity in the commercial preparation of phosphorylase kinase.

Assay of cyclic AMP. Cell suspension (0.75 ml) was rapidly pipetted into an equal volume of ice-cold 1 N perchloric acid, and proteins were removed by centrifugation. The supernatant fraction (1 ml) was removed and neutralized with 0.043 ml of 5 M KOH in 0.5 M triethanolamine. Potassium perchlorate was

* Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and NBTI, *p*-nitrobenzylthioinosine.

removed by centrifugation. The supernatant fraction was acidified, a tracer amount of [^{3}H]cyclic AMP was added as a marker of recovery, and the solution was purified on a column of Dowex 50W (H^+ form) [24]. The purified samples were then assayed for cylic AMP by radioimmunoassay following acetylation [25].

Materials. Dipyridamole was a gift from Boehringer-Ingelheim Ltd. Adenosine was obtained from P-L Biochemicals, and nitrobenzylthioinosine (NBTI) from Calbiochem Behring; theophylline, phenylephrine, 2-chloroadenosine, 2'-deoxyadenosine, and 5'-deoxyadenosine were from Sigma; and 5'-chloro-5'-deoxyadenosine was from Dr. John Palmer. 5'-*N*-Ethylcarboxamido-5'-deoxyadenosine (NECA) was prepared by Dr. S. Doctrow in this laboratory. Triton X-100, Amberlite (MB-1A), cyclic AMP, amyloglucosidase (type IV), and phosphorylase kinase from rabbit skeletal muscle were from Sigma. Glucose-6-phosphate dehydrogenase and phosphoglucomutase were from Boehringer-Mannheim. [2,8-^{3}H]Cyclic AMP and 2'-*O*-succinyl-[^{125}I]iodotyrosine methylester of cyclic AMP were from New England Nuclear. Goat anti-cyclic AMP antiserum, normal goat serum, and rabbit antigoat antiserum were obtained from Research Products International.

RESULTS

Incubation of hepatocytes prepared from fed rats in glucose-free medium was associated with a sustained release of glucose by the cells, as has been described previously [26]. Inclusion of 5'-chloro-5'-deoxyadenosine accelerated the rate of glucose release and decreased the glycogen content in a dose-dependent manner (Fig. 1). Similar findings were obtained with the R receptor agonist 5'-deoxyadenosine but not with 2'-deoxyadenosine, an adenosine analogue without R receptor agonist activity [27] (Fig. 2). The effect of 5'-chloro-5'-deoxyadenosine was not diminished by NBTI, a potent inhibitor of nucleoside transport [28], indicating that uptake was not necessary for the analog to exert its effect (results not shown). Theophylline, a blocker of adenosine R receptors [4], markedly attenuated the effect of 5'-chloro-5'-deoxyadenosine on glucose release by hepatocytes (Fig. 3). These results are consistent with the activation of an external, plasma membrane adenosine receptor. Other R receptor agonists such as 2-chloroadenosine and NECA showed similar potencies with respect to glucose release (results not shown). However, adenosine itself showed no stimulation of glucose release when this was measured after 40 min of incubation, even when the adenosine concentration was 160 μM (results not shown).

Hepatocytes initially kept at 0° were incubated at 37° for 20 min. 5'-Chloro-5'-deoxyadenosine (20 μM) was then added, and samples were assayed for phosphorylase *a* and total phosphorylase activities (Fig. 4). Phosphorylase *a* activity was very low in chilled hepatocytes but rose within 5 min to approximately 50% of the total activity. Within about 1 min of adding 5'-chloro-5'-deoxyadenosine, phosphorylase *a* activity increased to a value close to the total activity which could be obtained by activation of broken cell extracts using rabbit skeletal muscle phosphorylase *b* kinase (see Experimental Procedures). Moreover, the increase in activity was sustained for at least 4 min. Hepatocytes isolated from a fed rat were preincubated at 37° for 15 min. Either 20 μM adenosine or 20 μM 5'-chloro-5'-deoxyadenosine was then added, and serial samples were taken for phosphorylase assays (Fig. 5). Phosphorylase *a* activity rose to a sustained higher level with 5'-chloro-5'-deoxyadenosine, as predicted from the earlier experiment. With adenosine, a similar

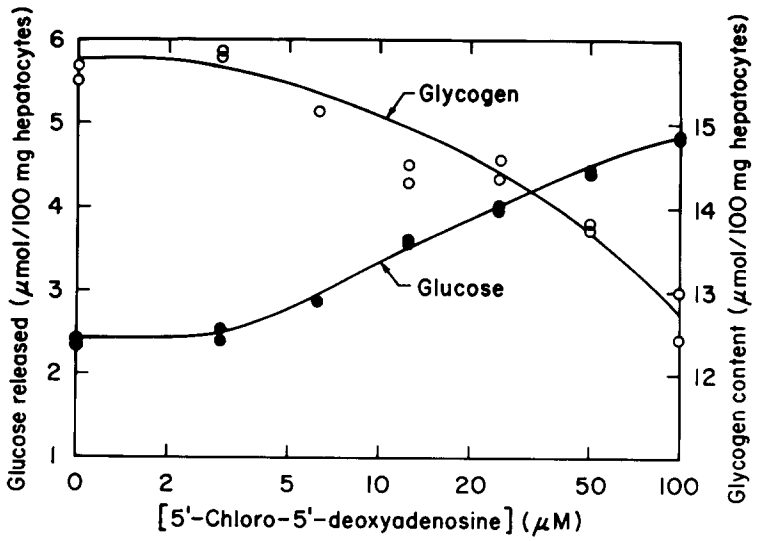

Fig. 1. Glycogen content of, and glucose output by, hepatocytes incubated in the presence of different concentrations of 5'-chloro-5'-deoxyadenosine. Hepatocytes (40 mg/ml) prepared from a fed rat were incubated in duplicate flasks in glucose-free Krebs–Henseleit bicarbonate buffer and 0.4% bovine serum albumin for 40 min.

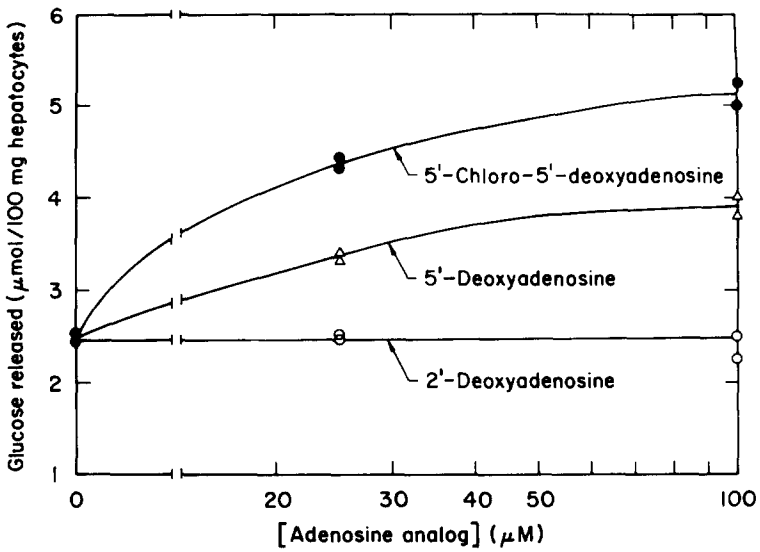

Fig. 2. Glucose output by hepatocytes incubated in the presence of different concentrations of three adenosine analogues. The conditions were as described in the legend of Fig. 1.

rise in activity was observed, but this rise was not sustained and fell back to control levels within 5 min.

A dose-dependent increase in phosphorylase activity was found 1 min after the addition of adenosine. The activation varied somewhat from preparation to preparation. In Fig. 6, half-maximum activation occurred at 3 μM adenosine. NBTI showed a slight stimulating effect. Dipyridamole failed to inhibit glucose release or phosphorylase activation (results not shown). On the other hand, 125 μM theophylline inhibited the rise in activiy at low, but not at high adenosine concentrations (Fig. 6). Phosphorylase activation by 5'-chloro-5'-deoxyadenosine was observed to be the same in hepatocytes incubated in regular or calcium-depleted medium (results not shown). When cyclic AMP levels of the hepatocytes were determined 1 min after the addition of adenosine, a dose-dependent rise was observed, with basal levels of approximately 0.66 pmole cyclic AMP/mg of cells rising to maximum levels of approximately 1.5 pmoles cyclic AMP/mg cells (Fig. 7).

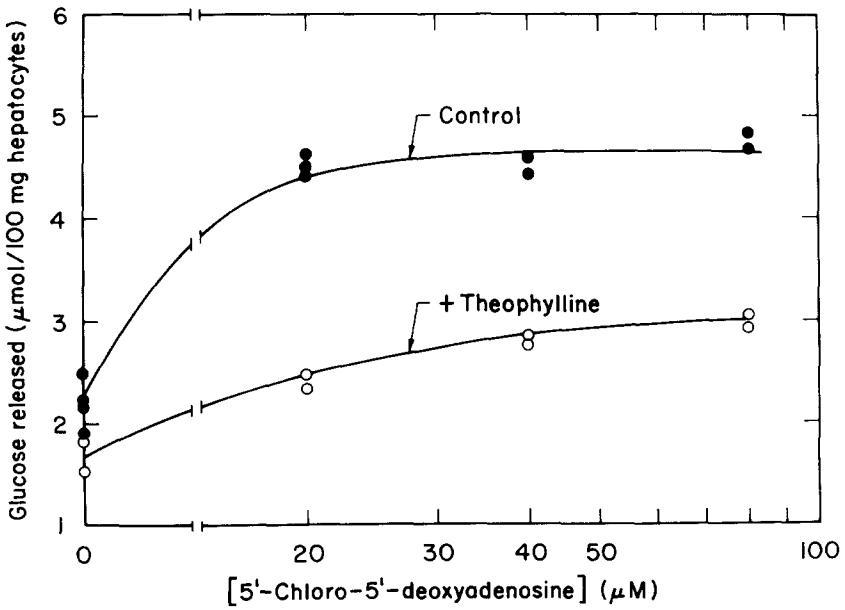

Fig. 3. Effect of theophylline on 5'-chloro-5'-deoxyadenosine-stimulated glucose output by hepatocytes. Hepatocytes from a fed rat were incubated in the absence or the presence of 1 mM theophylline. The conditions were as described in the legend of Fig. 1.

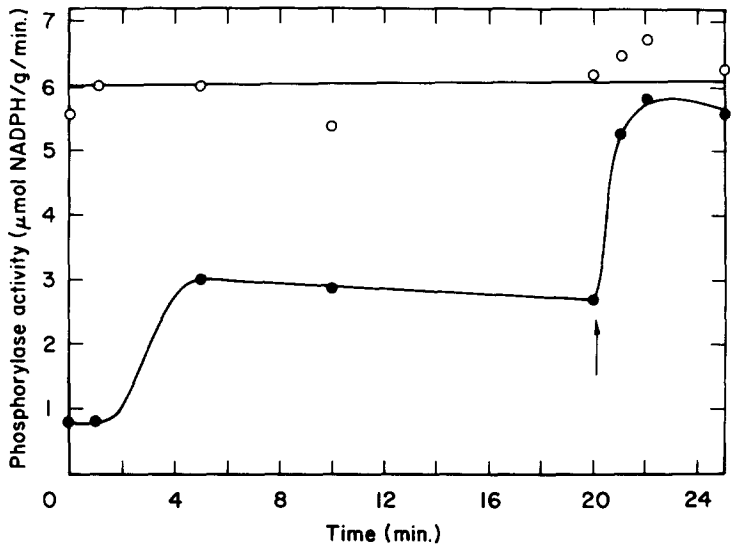

Fig. 4. Effects of warming and of 5′-chloro-5′-deoxyadenosine on phosphorylase activity of hepatocytes. Hepatocytes prepared from a fed rat (40 mg/ml) were kept on crushed ice in 20 mM glucose Krebs–Henseleit bicarbonate buffer and 0.4% bovine serum albumin under 95% oxygen and 5% CO_2 in a stoppered Erlenmeyer flask. At zero time the flask was transferred to a shaking water bath at 37° and gassed continuously while samples were taken at intervals. 5′-Chloro-5′-deoxyadenosine (20 μM) was added immediately after the 20-minute sample (arrow). Key: (●) phosphorylase *a* activity; and (○) total phosphorylase activity.

DISCUSSION

Our results confirm and extend the findings of Bartrons *et al.* [13] who observed an activation of liver phosphorylase by micromolar concentrations of adenosine and certain of its analogues. Fain and Shepherd [12] concluded that micromolar concentrations of adenosine have no appreciable effect on cyclic AMP metabolism or glycogenolysis by hepatocytes. These authors used glucose release as the criterion of an adenosine effect. Even relatively high concentrations of adenosine are metabolized

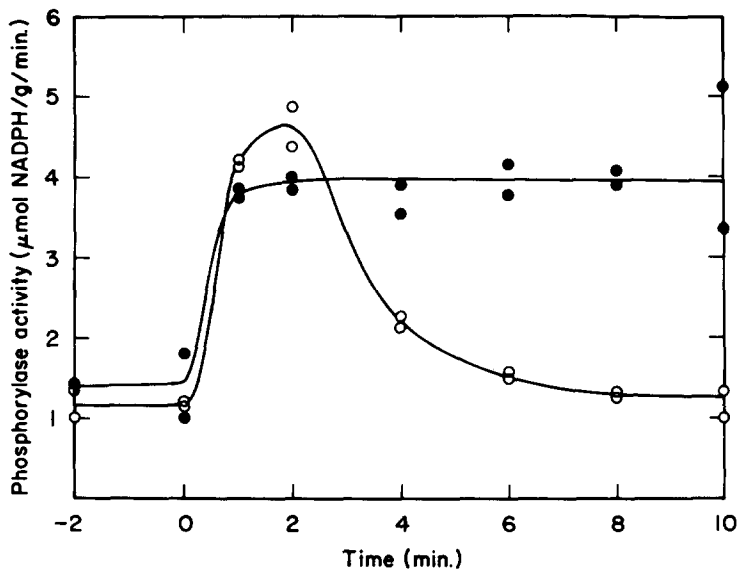

Fig. 5. Time-course of phosphorylase activation by adenosine and 5′-chloro-5′-deoxyadenosine. Hepatocytes prepared from a fed rat were preincubated for 15 min at 37° in the medium described in the legend of Fig. 4. Two samples were withdrawn for the assay of basal phosphorylase *a* activity. Immediately after the second sample was taken, 20 μM adenosine (○) or 20 μM 5′-chloro-5′-deoxyadenosine (●) was added (zero time), and further samples were taken as indicated.

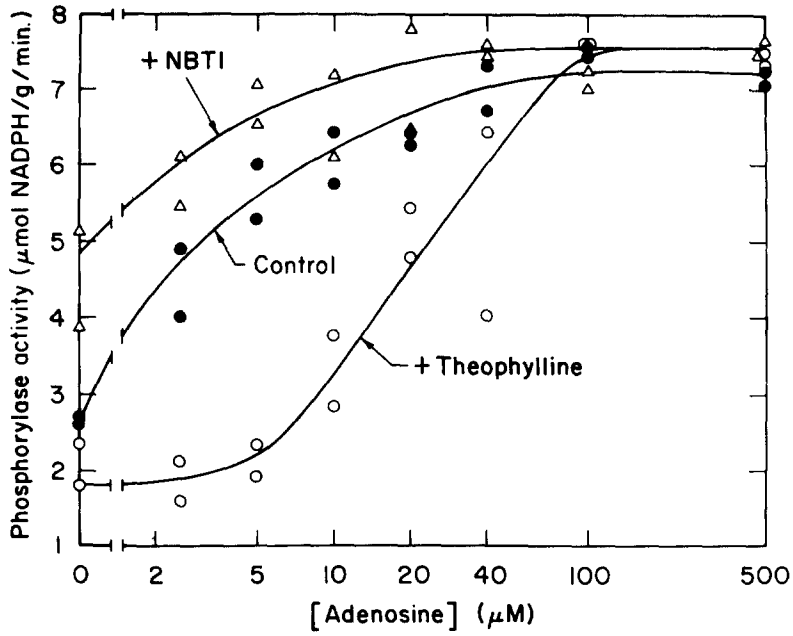

Fig. 6. Effect of nitrobenzylthioinosine and theophylline on adenosine-stimulated phosphorylase activation in hepatocytes. Hepatocytes prepared from a fed rat were preincubated at 37° in the medium described in Fig. 4, either without any supplementation, or with 20 μM nitrobenzylthioinosine, or with 125 μM theophylline for 10 min. Adenosine was then added as indicated, and samples were withdrawn 1 min later for phosphorylase *a* determination.

very rapidly by hepatocytes [3, 9, 12, 13]. Figure 5 shows that the effect of adenosine on phosphorylase is transient, probably because of its rapid removal. The transient modulation of phosphorylase activity by adenosine in response to changes in the energy metabolism of the hepatocyte may have a physiological counterpart in the intact liver. Fain and Shepherd [12] found that very high concentrations of adenosine prevent the stimulation of glycogenolysis by glucagon and epinephrine. This effect must be due to another process, perhaps related to the increase in adenine nucleotide concentrations which occurs in response to high concentrations of adenosine [3, 9–11].

Unlike adenosine, 5′-chloro-5′-deoxyadenosine produced a sustained increase in phosphorylase activity (Fig. 5). This compound is not deaminated (Naito and Lowenstein, unpublished observations)

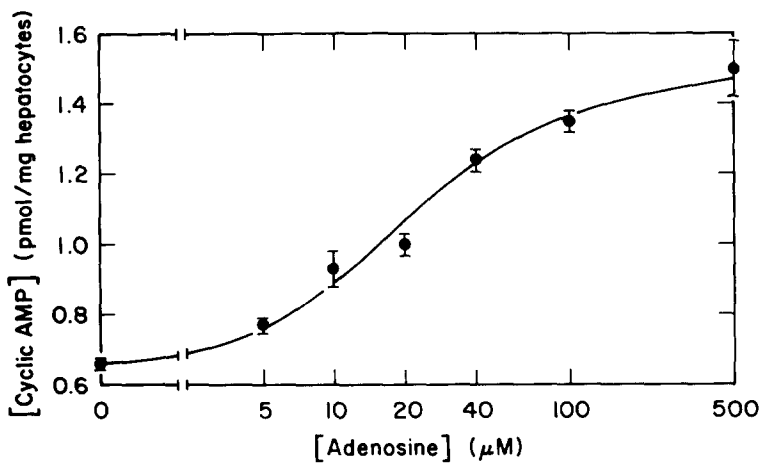

Fig. 7. Effect of adenosine on cyclic AMP content of hepatocytes. Hepatocytes (40 mg/ml) were preincubated at 37° in the medium described in Fig. 4 for 10 min. Adenosine was then added, and samples were withdrawn 1 min later for cyclic AMP determination. Results show the mean ± SE of duplicate incubations assayed in duplicate or quadruplicate.

and cannot be phosphorylated at the 5′-position; hence its effects persist. A similar explanation applies to other analogues modified at the 5′-position such as NECA and 5′-deoxyadenosine [29, 30]. Bartrons *et al.* [13] reported similar results with 2-chloroadenosine. 2′-Deoxyadenosine is not an R receptor agonist, but its failure to exert an effect could also have been due to deamination or other metabolic transformation. 2-Chloroadenosine and 5′-chloro-5′-deoxyadenosine were equally potent in their effects over a wide range of concentrations, reaching a maximum rate of glucose release at a concentration of 40 μM, with no further change in rate up to concentrations of 0.5 mM (data not shown). Similarly, the effect of a 1-min exposure to adenosine on phosphorylase showed no sign of diminution at high concentrations of adenosine (Fig. 5). This casts some doubt on the function of the P site, which has been postulated to mediate a decrease in adenylate cyclase activity [4], at least as far as this applies to hepatocytes.

NBTI and dipyridamole did not inhibit the stimulation of glucose release or phosphorylase activity by adenosine and its analogues. This is evidence that the adenosine effect studied by us was exerted at an extracellular site. Both transport inhibitors showed a slight stimulatory effect. To obtain stable, accurate concentrations in the incubation media, these compounds were added as concentrated solutions in DMSO. DMSO (4%) itself stimulated glucose release; however, in the actual experiments DMSO concentrations did not exceed 0.8%, and this had no measurable effect.

The inhibitory effect of theophylline, when present in approximately a 20-fold molar excess over adenosine, is additional evidence that the effect of adenosine is due to activation of external R receptors. The failure of theophylline to inhibit at higher concentrations of agonist (Fig. 5) is typical of methylxanthine inhibition, which is of the competitive type [30].

Berne *et al.* [31] showed that hypoxic hepatocytes release adenosine into the incubation medium. Moreover, hypoxia markedly stimulates liver phosphorylase activity [32, 33]. Fructose treatment causes a rapid depletion of hepatic ATP without a corresponding rise in AMP [34–37]; this could be due to the hydrolysis of AMP to adenosine. Adenosine thus formed could stimulate R receptors, leading to a rise in cyclic AMP. Such a rise was observed by Miller [38]. While it seems clear that an adenosine-mediated rise in cyclic AMP is not the sole cause of the fructose-induced increase in phosphorylase activity [37], this does not rule out the possibility that adenosine could, under more physiological conditions, play an important role. Indeed, the present results, taken with those of Bartrons *et al.* [13] and the report of adenosine release by hepatocytes rendered hypoxic [31], provide evidence that adenosine modulates cyclic AMP levels as part of a feedback control of hepatic ATP levels. ATP depletion results in an increase in the adenosine concentration. This activates the adenylate cyclase system and accelerates glycogenolysis, making more hexose available for energy production and ultimately for increased ATP formation.

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