

# Stimulation of glucose production from glycogen by glucagon, noradrenaline and non-degradable adenosine analogues is counteracted by adenosine and ATP in cultured rat hepatocytes

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The glycogenolytic potency of adenosine and ATP was studied in adult rat hepatocytes and compared with the action of glucagon and noradrenaline. In cells cultured for 48 h, adenosine and ATP as well as their analogues 2-chloroadenosine, phenylisopropyladenosine, *N*-ethylcarboxamidoadenosine and  $\beta,\gamma$ -methylene-substituted ATP (p[CH<sub>2</sub>]<sub>ppA</sub>) increased glycogen phosphorylase *a* to levels indistinguishable from those obtained by the addition of glucagon or noradrenaline. The P<sub>1</sub> receptor antagonist 8-phenyltheophylline abolished the activation of phosphorylase by adenosine and by p[CH<sub>2</sub>]<sub>ppA</sub>, but not that by ATP. Protein kinase A was activated by p[CH<sub>2</sub>]<sub>ppA</sub> and ATP via their breakdown to adenosine. [<sup>14</sup>C]Glucose production from glycogen was stimulated only 3-fold by ATP and adenosine, compared with a 7-fold increase produced by the hormones. Stimulation of glucose production by glucagon or noradrenaline was almost completely abolished by ATP or adenosine, with half-maximal effects at around 10  $\mu$ M. The non-degradable adenosine analogues were equipotent with glucagon with respect to stimulation of glucose production, and their action was also inhibited by adenosine. ATP and p[CH<sub>2</sub>]<sub>ppA</sub>, which were both degraded to adenosine, showed comparable metabolic effects, whereas the  $\alpha,\beta$ -methylene analogue was without biological action and also was not degraded to adenosine. In the presence of the adenosine transport inhibitor nitrobenzyl thioinosine (NBTI), adenosine exerted an increased glycogenolytic potency, reaching 80% of the maximal stimulation obtained by glucagon. The glucagon-antagonistic effect of adenosine could be completely abolished by NBTI, but was not affected by phenyltheophylline. It is concluded that, in the hepatocyte culture system, adenosine and ATP decrease the catalytic efficiency of phosphorylase *a* through signals arising from their uptake into the cell.

## INTRODUCTION

The activation of hepatic glycogenolysis by adenosine and ATP has recently received much attention. It is well documented that both purines activate glycogen phosphorylase [1–4] and increase glucose production in perfused livers from fed rats, an action comparable with the effects of noradrenaline and glucagon and of hepatic nerve stimulation [5–7]. The effects of adenosine are thought to be mediated by an increase in cyclic AMP concentration via P<sub>1</sub> receptors [3,8,9], whereas the signal chain initiated by ATP appears to involve the breakdown of phosphatidylinositol to InsP<sub>3</sub> and mobilization of intracellular Ca<sup>2+</sup> via P<sub>2y</sub> receptors in a cyclic AMP-independent manner [10–15]. A number of studies with hepatocyte suspensions report ATP- and adenosine-dependent activation of glycogen phosphorylase [3,4,13,15,16], but very rarely has glucose production also been determined in this isolated cell system. There are few and conflicting reports on the glycogenolytic potency of adenosine and ATP in hepatocyte suspensions. Basal glycogenolysis has been reported to be not affected [17], slightly decreased [18] or increased [19,20] by these purines.

During an investigation into the interaction of ATP and insulin in their effects on glycogen metabolism, we found that in the system used, the primary cultured adult hepatocyte, ATP inhibited glucose production from glycogen and at the same time activated glycogen phosphorylase to the same degree as did glucagon. The present study investigates this paradoxical action of adenosine and ATP on glycogen metabolism in hepatocyte cultures. It is shown that, depending on the experimental

conditions, ATP and adenosine inhibited or stimulated glucose production. Stimulation never reached the degree obtained with glucagon or noradrenaline, the actions of which were antagonized severely by the two purines. It is suggested that the stimulatory receptor-dependent actions of adenosine and ATP were counteracted by signals arising from the cellular uptake of the purines.

## EXPERIMENTAL

### Materials

Enzymes, cell culture media and adenosine were obtained from Boehringer (Mannheim, Germany); bovine insulin, glucagon and noradrenaline were from Serva (Heidelberg, Germany), dexamethasone, 8-phenyltheophylline, ATP and all ATP and adenosine analogues were from Sigma (Taufkirchen, Germany); and collagenase type I was from Worthington (Biochrom, Berlin, Germany). D-[U-<sup>14</sup>C]Glucose and [ $\gamma$ -<sup>32</sup>P]ATP were from New England Nuclear (Dreieich, Germany), and D-*myo*-[2-<sup>3</sup>H]inositol was from Amersham (Braunschweig, Germany). Stock solutions of phenyltheophylline (10 mM) and nitrobenzyl thioinosine (NBTI; 100 mM) were made in 0.01 M-NaOH and dimethyl sulphoxide respectively.

### Cell culture

Male Wistar rats (180–250 g) were kept on a 12 h light/12 h dark rhythm and were allowed free access to the standard diet ssniff R 15 (ssniff, Soest, Germany). Hepatocytes were isolated

Abbreviations used: PIA, *N*-(1-2-phenylisopropyl)adenosine; NECA, 5'-*N*-ethylcarboxamidoadenosine; pp[CH<sub>2</sub>]<sub>ppA</sub>, adenosine 5'-[ $\alpha,\beta$ -methylene]-triphosphate; p[CH<sub>2</sub>]<sub>ppA</sub>, adenosine 5'-[ $\beta,\gamma$ -methylene]triphosphate; NBTI, nitrobenzyl 6-thioinosine; EBM, Eagle's basal medium.

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by recirculating collagenase perfusion *in situ* and cultured in M199 medium on 60 mm plastic dishes [21]. For the first 3 h (attachment phase) medium contained 4% newborn calf serum, 1 nM-insulin and 0.1  $\mu$ M-dexamethasone. After the first medium change (2.5 ml/dish) serum was omitted and the cells were cultured for the next 20 h in the presence of 0.1  $\mu$ M-dexamethasone and 1 nM-insulin. From 24 to 46 h cells were cultured with 0.1  $\mu$ M-dexamethasone, 10 nM-insulin and either 5 mM- (normal M199 medium) or 20 mM-glucose (M199 plus 15 mM additional glucose). For the determination of glucose production from glycogen, glycogen was labelled with [ $^{14}$ C]glucose (1.5  $\mu$ Ci/ml) from 24 to 46 h. The gas atmosphere contained 5% CO<sub>2</sub>, 17% O<sub>2</sub> and 78% N<sub>2</sub>.

### Cell experiments

Since the culture medium M199 contains 1.6  $\mu$ M-ATP, the cell experiments were performed in Eagle's basal medium (EBM), which contains no added nucleotides. After 46 h, dishes were washed twice and then incubated in EBM (2 ml/dish) containing 2 mM added lactate. For the glycogen synthesis experiments, medium was additionally supplemented with [ $^{14}$ C]glucose (0.8  $\mu$ Ci/dish) and 5 mM extra glucose. After a 30 min pre-incubation, zero-time samples were taken and the experiment was started by the addition of agonists to the dishes. The incubation was terminated by rapidly aspirating the medium and immersing the dishes in liquid N<sub>2</sub>. For the determination of  $^{14}$ CO<sub>2</sub> production, cells received EBM without bicarbonate and each dish was transferred to an airtight incubation chamber (37 °C) filled with air. These experiments were terminated by injecting 0.5 ml of 1 M-HCl through a rubber stopper into the medium. Dishes for the determination of metabolites were washed three times with ice-cold 0.9% NaCl before freezing. It was verified that the quick wash procedure ( $\leq 20$  s) did not alter the concentration of metabolites.

### Analytical procedures

Rates of glucose formation from glycogen and of glycolysis were estimated by the rates of glucose and lactate release into the culture medium. Labelled glucose was separated from labelled lactate by chromatography of 100  $\mu$ l of medium on Dowex 1  $\times$  8 (formate form), as outlined in [22]. The rate of glucose production was calculated from the specific radioactivity of glycogen [23], and is expressed as glycosyl units  $\cdot$  mg of DNA<sup>-1</sup>. The rate of glycogen synthesis was determined by extracting and quantifying the [ $^{14}$ C]glycogen from one dish according to Fleig *et al.* [23].  $^{14}$ CO<sub>2</sub> was trapped in 0.5 ml of 2 M-KOH placed in a reservoir within the incubation chamber before the experiment.

Cells in single dishes were processed for enzyme determination. The active form of glycogen phosphorylase in cytosolic extracts was determined according to [24]. Protein kinase A was assayed with 100  $\mu$ M-Kemptide as substrate as described [22,25].

Glycogen was determined after treatment of cell homogenates with amyloglucosidase [23] and subsequent enzymic determination of glucose.

For the determination of inositol phosphates, cells were incubated from 30 to 48 h with *myo*-[2- $^3$ H]inositol (10  $\mu$ Ci/ml of medium; 20  $\mu$ Ci/dish). Medium was removed and the monolayers were washed with four changes of medium. They then received 2 ml of medium containing 2 mM-lactate and 0.1  $\mu$ M-dexamethasone and preincubated for 30 min. During the last 10 min, 10 mM-LiCl was added, in the presence of which inositol monophosphate dephosphorylation is inhibited and inositol is trapped as inositol phosphates. Agonists were then added and cells were harvested 1–10 min later by aspiration of the medium and addition of 500  $\mu$ l of 10% trichloroacetic acid. After centrifugation and extraction of trichloroacetic acid by ether,

inositol phosphates were separated on Dowex 1  $\times$  8 (formate form) columns as described elsewhere [26]. DNA was estimated as detailed elsewhere [27].

For the determination of extracellular purines, medium was deproteinized with trichloroacetic acid (final concentration 8%), then trichloroacetic acid was extracted with ether and the pH was adjusted to 6.0 with KOH. Medium containing *N*-(1-2-phenylisopropyl)adenosine (PIA) was deproteinized with 0.3 M-HClO<sub>4</sub> and neutralized with potassium bicarbonate. Separation and quantification was achieved by h.p.l.c. (Merck-Hitachi) at room temperature. ATP, ATP analogues, ADP, AMP and adenosine were chromatographed on a ET 250/8/4 Nucleosil 5 C<sub>18</sub> column (Machery and Nagel, Düren, Germany). They were eluted with a stepwise gradient of buffer A (0.1 M-KH<sub>2</sub>PO<sub>4</sub>, pH 6.0) and buffer B (0.1 M-KH<sub>2</sub>PO<sub>4</sub>, pH 6.0, with 10% methanol). Chloroadenosine was eluted with buffer B and PIA with 50 mM-KH<sub>2</sub>PO<sub>4</sub>, pH 6.0 (containing 50% methanol), from Merck RP C<sub>18</sub> columns (125-4). 5'-*N*-Ethylcarboxamidoadenosine (NECA) was eluted with 50 mM-KH<sub>2</sub>PO<sub>4</sub>, pH 6.0, containing 25% methanol from a Merck RP C<sub>18</sub> column (250-4).

### RESULTS

Cells used in this study contained different amounts of total glycogen due to different cell culture conditions. When cells were cultured with 5 mM-glucose (original M199 medium), glycogen content was  $5.1 \pm 0.17$   $\mu$ mol of glucose  $\cdot$  mg of DNA<sup>-1</sup> at the start of the experiment. Culture with 20 mM-glucose from 24–46 h increased their glycogen amount to  $67.4 \pm 5.0$   $\mu$ mol of glucose  $\cdot$  mg of DNA<sup>-1</sup>. Irrespective of culture conditions, experiments were carried out with 5 mM-glucose.

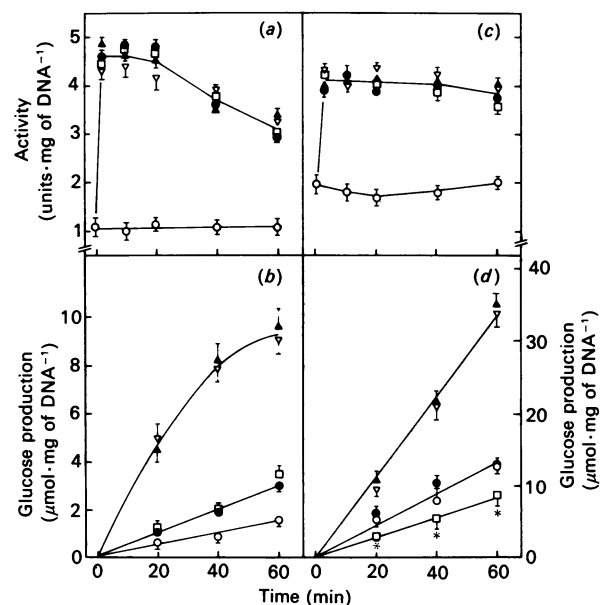


Fig. 1. Activation of phosphorylase (a and c) and stimulation of glucose production (b and d) by hormone and purine agonists

Cells were cultured in serum-free medium for 46 h as described in the Experimental section. Cellular glycogen was labelled from 24–46 h with either 5 mM- (a and b) or 20 mM- (c and d) [ $^{14}$ C]glucose. The experiment was then carried out with 5 mM-glucose and 2 mM-lactate in EBM and started by the addition of 10 nM-glucagon (▲), 10  $\mu$ M-noradrenaline (▽), 100  $\mu$ M-adenosine (●) or 100  $\mu$ M-ATP (□). Data are means  $\pm$  S.D. from four different cell cultures. \*Significantly different from the control ( $P < 0.025$ ); Student's *t* test for paired data.

### Influence of adenosine and ATP on glycogen metabolism in cultured hepatocytes

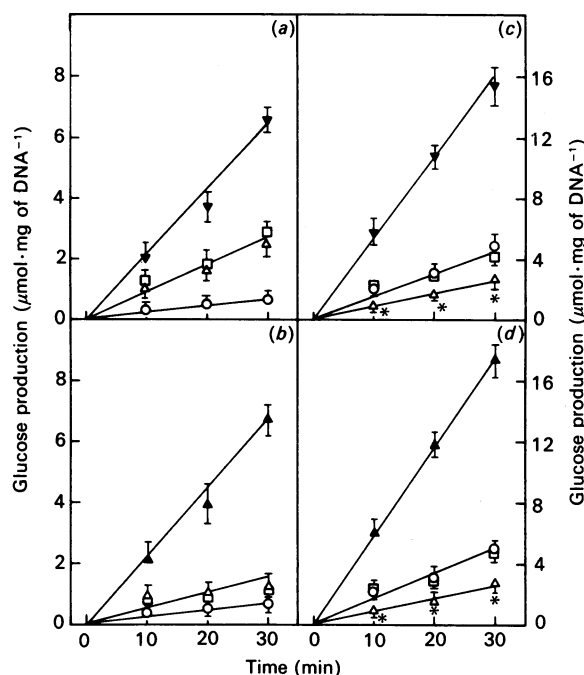
Basal glucose production from prelabelled glycogen was linear for at least 60 min in cells cultured with either 5 or 20 mM-glucose (Figs. 1b and 1d) and amounted to 1.7 and 12.6  $\mu\text{mol}$  of glycosyl units  $\cdot \text{h}^{-1} \cdot \text{mg}$  of DNA $^{-1}$  respectively. Glycogen phosphorylase *a* activity was enhanced in cells which had been cultured with 20 mM-glucose compared with cells cultured with 5 mM-glucose (Figs. 1a and 1c). Addition of 10 nM-glucagon, 10  $\mu\text{M}$ -noradrenaline, 100  $\mu\text{M}$ -ATP or 100  $\mu\text{M}$ -adenosine increased phosphorylase *a* activity 4-fold (5 mM-glucose) or 2-fold (20 mM-glucose), irrespective of whether the agonist was a hormone or a purine. However, this uniform effect on phosphorylase activity did not result in quantitatively similar increases of glucose production. Whereas glucagon and noradrenaline increased basal glucose output by 7-fold in cells cultured with 5 mM-glucose, ATP and adenosine only doubled the basal rate (Fig. 1b). The difference in response was even more pronounced in cells cultured with 20 mM-glucose. Glucagon and noradrenaline increased basal glucose output by 2.7-fold, adenosine elicited no effect and ATP significantly decreased glucose production, by 34% (Fig. 1d). If, however, glucose production was determined in the presence of 20 mM-glucose instead of 5 mM-glucose, phosphorylase activity was lowered to values of around 1.0 unit  $\cdot \text{mg}$  of DNA $^{-1}$ , and the rate of glucose formation was decreased and could again be increased by ATP and adenosine (results not shown). Thus the degree of activation of phosphorylase and of glucose production in the basal state determined the degree to which the purines increased (adenosine, ATP) or decreased (ATP) glucose production.

To test whether adenosine and ATP might actually fully activate glycogen breakdown but then channel glucose 6-phosphate preferentially into lactate, CO<sub>2</sub> or back into glycogen, lactate and CO<sub>2</sub> production as well as glycogen synthesis were determined (Table 1). Glycogen synthesis, which was measured with 10 mM- instead of 5 mM-glucose in order to obtain measurable rates within 1 h, was decreased by adenosine and ATP (75% inhibition), as was the production of glycogen-derived lactate (45% inhibition). CO<sub>2</sub> formation was not affected by adenosine, ATP or noradrenaline, whereas it was increased by glucagon by about 50%. Total glycogen content in cells cultured with 20 mM-glucose was not decreased by adenosine or ATP (results not shown). Thus the poor glycogenolytic effect (as measured by glucose formation) of adenosine and ATP in the

**Table 1. Agonist-induced changes in glycogen synthesis and degradation in cultured hepatocytes**

Cells were cultured for 46 h with 5 mM-glucose as described in the Experimental section. During the 1 h short-term experiment when agonists were present, medium contained 5 mM-glucose, except for the determination of glycogen synthesis, which was measured with 10 mM-glucose. Concentrations were 10 nM-glucagon, 10  $\mu\text{M}$ -noradrenaline, 100  $\mu\text{M}$ -ATP and 100  $\mu\text{M}$ -adenosine. Values are means  $\pm$  s.d. from three different cell cultures.

Agonist	Glycogen synthesis ( $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg}$ of DNA $^{-1}$ )	Glycogen degradation ( $\mu\text{mol}$ of glycosyl units $\cdot \text{h}^{-1} \cdot \text{mg}$ of DNA $^{-1}$ ) to:		
		Glucose	Lactate	CO <sub>2</sub>
Control	2.04 $\pm$ 0.17	1.7 $\pm$ 0.29	0.37 $\pm$ 0.02	0.49 $\pm$ 0.02
Glucagon	0.05 $\pm$ 0.01	9.6 $\pm$ 0.85	0.24 $\pm$ 0.02	0.73 $\pm$ 0.01
Noradrenaline	0.18 $\pm$ 0.04	9.4 $\pm$ 0.74	0.16 $\pm$ 0.04	0.56 $\pm$ 0.01
ATP	0.60 $\pm$ 0.12	3.4 $\pm$ 0.41	0.20 $\pm$ 0.02	0.49 $\pm$ 0.02
Adenosine	0.41 $\pm$ 0.10	3.4 $\pm$ 0.35	0.20 $\pm$ 0.04	0.47 $\pm$ 0.01



**Fig. 2. Inhibition of glucagon- and noradrenaline-stimulated glucose production by adenosine and ATP**

Cellular glycogen was labelled and the experiment was performed essentially as described in the legend to Fig. 1, with the difference that 100  $\mu\text{M}$ -adenosine or 100  $\mu\text{M}$ -ATP was added 10 min before addition of 10  $\mu\text{M}$ -noradrenaline (a and c) or 10 nM-glucagon (b and d). (a, c)  $\circ$ , Control;  $\blacktriangledown$ , noradrenaline;  $\square$ , noradrenaline + adenosine;  $\triangle$ , noradrenaline + ATP; (b, d)  $\circ$ , control;  $\blacktriangle$ , glucagon;  $\square$ , glucagon + adenosine;  $\triangle$ , glucagon + ATP. Data are means  $\pm$  s.d. from three different cultures; \*significantly different from the control ( $P < 0.025$ ); Student's *t* test for paired data.

presence of a high phosphorylase *a* level cannot be explained in part by increased glycolytic and respiratory degradation of glycogen-derived glucose 6-phosphate.

These data led us to suggest that activation of glycogen breakdown by cyclic AMP-dependent (adenosine) and  $\text{InsP}_3$ -dependent (ATP) mechanisms was counteracted by other purine-elicited intracellular signals. Therefore we tested whether the glycogenolytic effects of glucagon (cyclic AMP-dependent) and noradrenaline ( $\text{InsP}_3$ -dependent) were influenced by the simultaneous addition of adenosine and ATP (Fig. 2). Adenosine or ATP (100  $\mu\text{M}$ ) completely abolished the 6–9-fold activation of glucose output by noradrenaline (Figs. 2a and 2c) and glucagon (Figs. 2b and 2d). Adenosine and ATP did not diminish the activation of glycogen phosphorylase by the hormones. When cells had been cultured with 20 mM-glucose, ATP but not adenosine lowered glucose production below the control rate, even in the presence of the hormones (Figs. 2c and 2d). Thus ATP and adenosine may generate intracellular signals which counteract hormonal activation of glycogen breakdown, irrespective of the hormonal signal transduction mode.

#### Dose-response curves

When basal glucose production was stimulated with increasing concentrations of ATP, a biphasic response was observed (Fig. 3a). Maximal stimulation was 3.5–4-fold at concentrations between 10 and 20  $\mu\text{M}$ -ATP. At higher concentrations the degree of stimulation decreased again and reached a final value (approx. 3-fold) at a concentration of approx. 100  $\mu\text{M}$ . Thus the dose-response curve for ATP mirrors the antagonism between the stimulatory and inhibitory signals. A maximal 3-fold stimulation

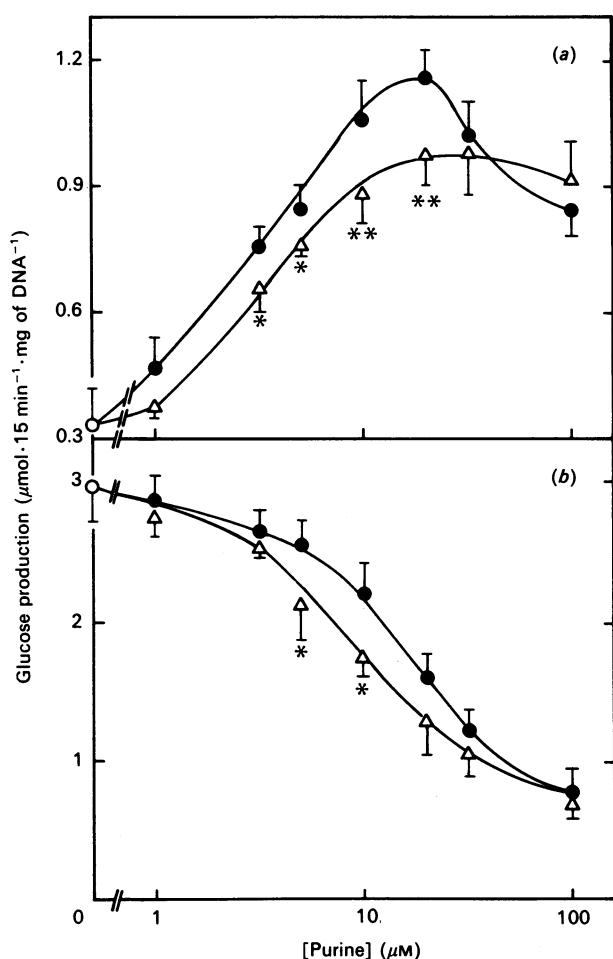


Fig. 3. Dose-response curves for the effects of adenosine ( $\Delta$ ) and ATP ( $\bullet$ ) on glucose production

Cells were cultured in serum-free medium for 46 h as described in the Experimental section. Cellular glycogen was labelled from 24–46 h with 5 mM- $^{14}\text{C}$ glucose and the experiment was carried out in the presence of 5 mM-glucose and 2 mM-lactate in EBM. The experiment was started by the addition of agonists, and  $^{14}\text{C}$ -glucose production was measured for 15 min. (a) Stimulation of basal glucose production; (b) inhibition of glucagon-stimulated glucose production. Glucagon concentration was 10 nM. Data are means  $\pm$  S.E.M. from three different cultures. \* $P < 0.05$ ; \*\* $P < 0.025$ : significant differences from the corresponding ATP values;  $t$  test for paired data.

was obtained with 20  $\mu\text{M}$ -adenosine which did not change further with increasing adenosine levels. Adenosine doses up to 20  $\mu\text{M}$  always led to a lower stimulatory response compared with the equivalent ATP doses. A higher inhibitory potential of adenosine is reflected in the dose-response curves in Fig. 3(b), which show the antagonistic effect of the purines on glucagon-stimulated glucose production. Compared with ATP, the curve for adenosine was shifted to lower purine concentrations. Half-maximal effective doses were 9  $\mu\text{M}$  for adenosine and 15  $\mu\text{M}$  for ATP.

#### Stimulation of glucose production by analogues of adenosine and ATP

The glycogenolytic potencies of the adenosine analogues 2-chloroadenosine, PIA and NECA were compared with those of adenosine and glucagon (Table 2). All analogues (100  $\mu\text{M}$ ) elicited greater metabolic responses than did adenosine. The effects of chloroadenosine and PIA were indistinguishable from that of

Table 2. Stimulation of glucose production by adenosine and adenosine analogues and rates of purine disappearance from the culture medium

Cells were cultured and the experiment was carried out as described in the legend to Fig. 3, except that glycogenolysis was measured for 30 min. Adenosine and its analogues were used at 100  $\mu\text{M}$  initial concentrations. Glucagon (10 nM) increased glucose production to 6.48  $\mu\text{mol} \cdot 30 \text{ min}^{-1} \cdot \text{mg}$  of DNA<sup>-1</sup> in this set of experiments. Data are means  $\pm$  S.D. from three or four different cultures; N.D., not determined.

Agonist	Glucose production ( $\mu\text{mol} \cdot 30 \text{ min}^{-1} \cdot \text{mg}$ of DNA <sup>-1</sup> )	Purine disappearance from the medium ( $\mu\text{mol} \cdot 30 \text{ min}^{-1} \cdot \text{mg}$ of DNA <sup>-1</sup> )
Control	0.60 $\pm$ 0.12	—
Adenosine	2.16 $\pm$ 0.07	2.22 $\pm$ 0.25
Chloroadenosine	6.62 $\pm$ 0.31	0.35 $\pm$ 0.08
PIA	6.63 $\pm$ 0.25	0.45 $\pm$ 0.10
NECA	5.69 $\pm$ 0.31	0.28 $\pm$ 0.07
Adenosine + chloroadenosine	2.54 $\pm$ 0.48	N.D.
Adenosine + PIA	2.23 $\pm$ 0.29	N.D.

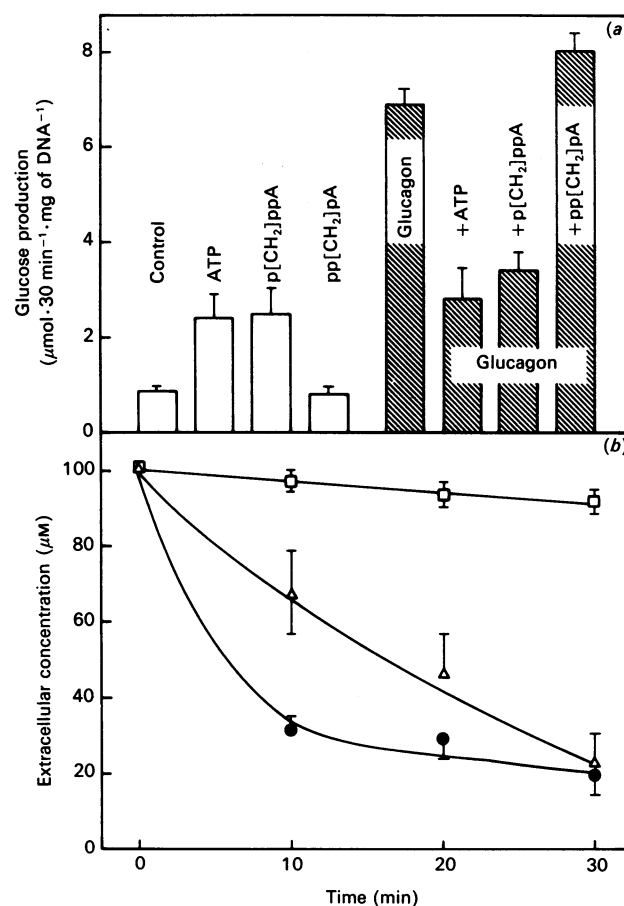


Fig. 4. Influence of ATP and ATP analogues on basal and glucagon-stimulated glucose production (a) and time course of purine disappearance from the culture medium (b)

Cell culture and experiment were carried out as described in the legend to Fig. 3. Glucose production was measured for 30 min. ATP and its analogues were used at 100  $\mu\text{M}$ ; glucagon at 10 nM. (b) ●, ATP;  $\Delta$ , p[CH<sub>2</sub>]ppA; □, pp[CH<sub>2</sub>]pA. Data are means  $\pm$  S.D. from four different cultures.

glucagon, and NECA was slightly less potent than the hormone (15%). Adenosine also abolished the actions of chloroadenosine and PIA on glucose production. The degree of phosphorylase activation was equal for all agonists tested. Whereas the extracellular concentration of adenosine decreased to 50% of the initial value after 30 min of incubation, degradation of the analogues did not exceed 5–10% during the same period, which suggested that the anti-glycogenolytic effect of adenosine originated in its degradation and/or uptake into the cell.

In contrast with the adenosine analogues, neither of the two ATP analogues tested, the methylene-substituted adenosine 5'-[ $\alpha,\beta$ -methylene]triphosphate (pp[CH<sub>2</sub>]pA) and adenosine 5'-[ $\beta,\gamma$ -methylene]triphosphate (p[CH<sub>2</sub>]ppA) were as potent as glucagon (Fig. 4); 100  $\mu$ M-p[CH<sub>2</sub>]ppA was equipotent with 100  $\mu$ M-ATP with respect to both its glycogenolytic and glucagon-antagonistic capacities. pp[CH<sub>2</sub>]pA did not increase glucose production, and the glucagon-stimulated glucose output was even slightly increased by this compound. The extracellular ATP concentration rapidly decreased by 70% during the first 10 min (Fig. 4b; [28]). The concentration of p[CH<sub>2</sub>]ppA fell at a slower

rate than did that of ATP (40% in 10 min), and the pp[CH<sub>2</sub>]pA level stayed almost constant (5% decrease in 30 min).

#### Involvement of cyclic AMP and InsP<sub>3</sub> in the actions of ATP and p[CH<sub>2</sub>]ppA

The methylene-substituted ATP analogues bind preferentially to P<sub>2x</sub> receptors [29]. Since the P<sub>2</sub> receptor found in adult rat hepatocytes is of the P<sub>2y</sub> type [15] the glycogenolytic action of p[CH<sub>2</sub>]ppA pointed to the involvement of cyclic AMP generated via activation of P<sub>1</sub> receptors by adenosine produced from p[CH<sub>2</sub>]ppA. Indeed, both 100  $\mu$ M-ATP and 100  $\mu$ M-p[CH<sub>2</sub>]ppA were degraded to adenosine in the cultured cells and led to a rapid activation of protein kinase A (Fig. 5). Maximal stimulation was reached within 2 min and amounted to an activity ratio (activity in the absence of cyclic AMP/activity in the presence of cyclic AMP) of 0.3 for ATP and 0.39 for p[CH<sub>2</sub>]ppA compared with a control value of 0.1. p[CH<sub>2</sub>]ppA was slightly more potent in stimulating protein kinase, although no quantitative difference between the two agonists was observed with respect to adenosine formation. pp[CH<sub>2</sub>]pA was not significantly degraded to adenosine and thus did not activate the protein kinase. Among the three purines tested, only ATP led to a pronounced formation of InsP<sub>3</sub>. Half of the maximal 16-fold increase of the InsP<sub>3</sub> level was obtained after cell exposure to 100  $\mu$ M-ATP for 1 min. The same concentrations of p[CH<sub>2</sub>]ppA and pp[CH<sub>2</sub>]pA merely doubled the InsP<sub>3</sub> content, consistent with data obtained with isolated hepatocytes [16].

The P<sub>1</sub> receptor antagonist 8-phenyltheophylline was used to test whether p[CH<sub>2</sub>]ppA exhibited its ATP-like action not through the increase of InsP<sub>3</sub> but through its degradation product adenosine and subsequent activation of the P<sub>1</sub> receptor (Table 3). Activation of glycogen phosphorylase and protein kinase A by 10  $\mu$ M-adenosine, which served as a positive control, was nearly completely reversed by 100  $\mu$ M-phenyltheophylline, as was activation of glucose production (results not shown). In contrast, 10  $\mu$ M-ATP led to a higher degree of phosphorylase activation (3.07 versus 2.05 units  $\cdot$  mg of DNA<sup>-1</sup>, in agreement with its higher glycogenolytic capacity; see Fig. 3) which could not be abolished by phenyltheophylline. However, the inhibitor

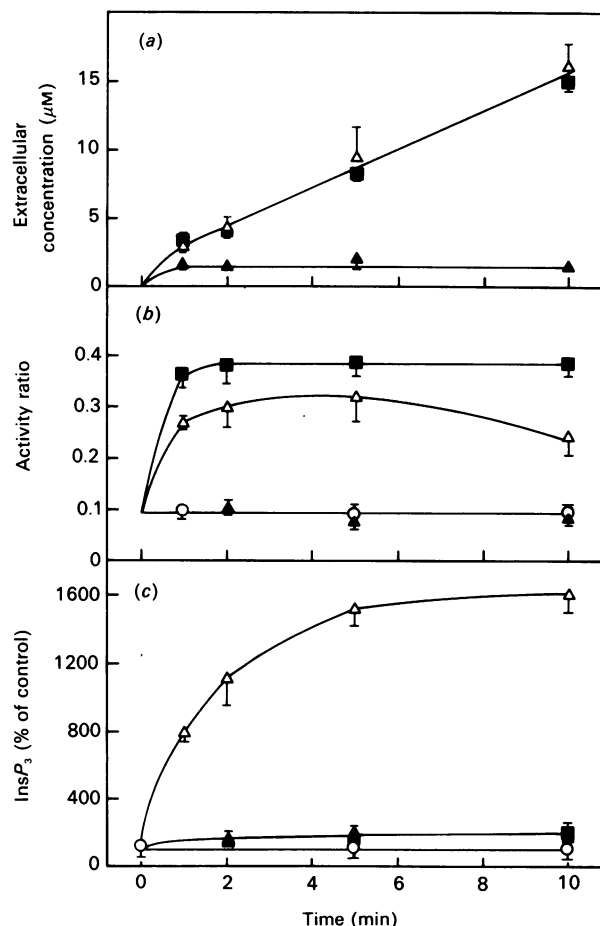


Fig. 5. Increase of intracellular signal components and accumulation of extracellular adenosine after addition of ATP and methylene-substituted ATP analogues to cultured hepatocytes

Cell culture and experiment were carried out as described in the legend to Fig. 3. The experiment was started by addition of 100  $\mu$ M-ATP or analogues. (a) Adenosine formation; (b) protein kinase A; (c) InsP<sub>3</sub> levels. ○, Control; △, ATP; ■, p[CH<sub>2</sub>]ppA; ▲, pp[CH<sub>2</sub>]pA. In (b), the activity ratio is given by (activity in the absence of cyclic AMP)/(activity in the presence of cyclic AMP). Data are means  $\pm$  S.D. from three or four different cultures.

Table 3. Effect of P<sub>1</sub> receptor blockade on the effects of adenosine, ATP and p[CH<sub>2</sub>]ppA on phosphorylase and protein kinase A activities

Cells were cultured for 46 h with 5 mM-glucose and the experiment was carried out as described in the Experimental section. 8-Phenyltheophylline (100  $\mu$ M) was added 10 min before the addition of agonists. After 2 min cells were harvested in liquid N<sub>2</sub>. Data are means  $\pm$  S.D. from four different cultures.

Agonist	Phenyl-theophylline	Phosphorylase <i>a</i> activity (units $\cdot$ mg of DNA <sup>-1</sup> )	Protein kinase A (activity ratio)
Control	-	0.56 $\pm$ 0.01	0.10 $\pm$ 0.01
Adenosine (10 $\mu$ M)	-	2.05 $\pm$ 0.18	0.23 $\pm$ 0.03
ATP (10 $\mu$ M)	+	0.75 $\pm$ 0.11	0.14 $\pm$ 0.01
ATP (10 $\mu$ M)	-	3.07 $\pm$ 0.21	0.14 $\pm$ 0.01
ATP (10 $\mu$ M)	+	2.70 $\pm$ 0.12	0.10 $\pm$ 0.01
ATP (100 $\mu$ M)	-	4.40 $\pm$ 0.14	0.28 $\pm$ 0.02
ATP (100 $\mu$ M)	+	4.40 $\pm$ 0.10	0.10 $\pm$ 0.01
p[CH <sub>2</sub> ]ppA (10 $\mu$ M)	-	1.19 $\pm$ 0.18	0.13 $\pm$ 0.01
p[CH <sub>2</sub> ]ppA (10 $\mu$ M)	+	0.63 $\pm$ 0.01	0.09 $\pm$ 0.01
p[CH <sub>2</sub> ]ppA (20 $\mu$ M)	-	1.51 $\pm$ 0.28	0.22 $\pm$ 0.01
p[CH <sub>2</sub> ]ppA (20 $\mu$ M)	+	0.67 $\pm$ 0.09	0.15 $\pm$ 0.02
p[CH <sub>2</sub> ]ppA (100 $\mu$ M)	-	3.70 $\pm$ 0.42	0.37 $\pm$ 0.03
p[CH <sub>2</sub> ]ppA (100 $\mu$ M)	+	1.76 $\pm$ 0.38	0.19 $\pm$ 0.03

decreased the slightly elevated kinase activity ratio. This latter effect could be more clearly demonstrated when 100  $\mu\text{M}$ -ATP was used. The receptor blocker completely reversed kinase activation without reversing the activation of glycogen phosphorylase. In analogy with adenosine, the modest activation of both phosphorylase and kinase by 10  $\mu\text{M}$ -p[CH<sub>2</sub>]ppA could be abolished by phenyltheophylline. At this concentration p[CH<sub>2</sub>]ppA increased basal glucose output by only 20% (results not shown). When higher p[CH<sub>2</sub>]ppA concentrations were used (20 and 100  $\mu\text{M}$ ) the inhibitor still strongly decreased the activating effects, although complete reversal could not be obtained with 100  $\mu\text{M}$ -phenyltheophylline. Thus p[CH<sub>2</sub>]ppA did not act by itself but only through its degradation product adenosine.

#### Inhibition of the glucagon-antagonistic action of adenosine and increase of the glycogenolytic potency of adenosine by NBTI

In contrast with its effective inhibition of the stimulatory action of adenosine, phenyltheophylline did not block the inhibitory effect of 20  $\mu\text{M}$ -adenosine on glucagon-stimulated glycogen breakdown ( $5.88 \pm 0.6$  versus  $6.25 \pm 1.2 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg}$  of DNA<sup>-1</sup>, with and without 200 nM-phenyltheophylline respectively,  $n = 4$ ). Therefore NBTI, an inhibitor of adenosine transport across the plasma membrane [30], was used to test whether the anti-glycogenolytic effect of adenosine was the result of adenosine uptake into the cell. The better-known transport inhibitor dipyridamole was not suitable for this study, since it strongly decreased basal and glucagon-stimulated glucose output (results not shown), probably due to its inhibitory effect on glucose transport [31]. In this set of experiments 20  $\mu\text{M}$ -adenosine only doubled basal glucose output; the latter was increased by 30% by dimethyl sulphoxide (0.1%), used as a solvent for NBTI (Table 4). NBTI (200  $\mu\text{M}$ ) alone also doubled the basal rate, but it did not affect the glucagon-stimulated rate, nor did it change the extracellular concentration of adenosine. Addition of both NBTI and adenosine led to a large increase in glucose production that reached 80% of the maximal increase obtained by glucagon. In the presence of NBTI the pronounced 50% decrease of the glucagon-stimulated rate by adenosine was no longer observed. NBTI had smaller effects on the stimulatory and inhibitory effects of ATP. Stimulation of glucose production was increased by approx. 50% and the inhibitory action of ATP on the glucagon response was antagonized by NBTI to very variable extents (10–60%, results not shown).

**Table 4. Effect of the adenosine transport inhibitor NBTI on adenosine action**

Cell culture and the experiment were carried out as described in the legend to Fig. 3. The cells received 200  $\mu\text{M}$ -NBTI 5 min before the addition of 20  $\mu\text{M}$ -adenosine and/or 10 nM-glucagon. Glucose production was measured for 30 min. Data are means  $\pm$  s.d. from three different cultures. Glucose production stimulated by glucagon amounted to  $6.1 \pm 0.8 \mu\text{mol} \cdot 30 \text{ min}^{-1} \cdot \text{mg}$  of DNA<sup>-1</sup>.

Agonist	NBTI	Glucose production from glycogen (% of glucagon-stimulated rate)
Control	-	14.8 $\pm$ 3.5
	+	32.0 $\pm$ 3.0
Adenosine	-	28.5 $\pm$ 1.1
	+	80.0 $\pm$ 7.9
Glucagon	-	100
	+	101.0 $\pm$ 3.5
Glucagon + adenosine	-	56.0 $\pm$ 2.1
	+	99.5 $\pm$ 4.0

## DISCUSSION

The data presented in this study (1) show that in cultured hepatocytes, adenosine and ATP can both increase and decrease glucose production; (2) indicate that in the culture system a purinergic increase in glycogen breakdown is mediated via the P<sub>1</sub> receptor (adenosine and ATP- and p[CH<sub>2</sub>]ppA-derived adenosine) and the P<sub>2</sub> receptor (ATP); (3) suggest that the inhibitory signal arises from uptake of adenosine into the cell and (4) strengthen the view that adenosine and ATP can exert metabolic effects which are not mediated via extracellular receptor activation in isolated cell systems.

It has been recognized for some time that extracellular adenosine and ATP, as well as many of their analogues, stimulate hepatic glycogenolysis. In contrast with many confirmatory reports, two groups demonstrated anti-glycogenolytic effects of adenosine [17] and ATP [18] working with isolated hepatocytes in suspension. Using the culture system of adult rat hepatocytes it was possible to demonstrate here both stimulatory and inhibitory effects of adenosine and ATP on glucose production. Stimulatory actions occurred when basal glucose production was very low (as is the case in hepatocytes after 48 h of culture with a glucose concentration of 5 mM; Fig. 1). Inhibitory actions were evident when basal glucose output had been increased by prior culture with 20 mM-glucose or by addition of glucagon and noradrenaline (Fig. 2) or by non-degradable adenosine analogues (Table 2). The stimulatory potency never reached that of glucagon, although the activation status of glycogen phosphorylase obtained by adenosine or ATP addition was not different from that obtained by the hormones. Thus it must be concluded that adenosine and ATP activate signal chains leading to activation of glycogen phosphorylase, and at the same time give rise to signals which inhibit the glycogenolytic action of phosphorylase *a* within the cell. This dual role is reflected in the dose-response curve for ATP (Fig. 3), which demonstrates that ATP (or ATP-derived adenosine) at higher concentrations inhibits its own stimulatory action and raises the question as to the mechanism involved.

Several lines of evidence suggest that the classical signal chain via the P<sub>1</sub> receptor does not elicit an additional negative feedback signal. (1) Adenosine analogues which act at the P<sub>1</sub> (A<sub>2</sub>) receptor and which are not significantly degraded or taken up by the cultured cells (Table 2) stimulated glucose production in a manner indistinguishable from the action of glucagon. (2) The inhibitory effects could not be abolished by phenyltheophylline, which did, however, antagonize the stimulatory response (Table 3). (3) Inhibition of adenosine uptake restored the attenuated stimulation of glucose production to a near-maximal value, and abolished inhibition of glucagon-stimulated glucose output (Table 4). From this we conclude that the inhibitory signal arises from the uptake of adenosine and from subsequent intracellular processing of the molecule. This latter hypothesis is corroborated by the finding of Fain & Shepherd [17] that 2',5'-dideoxyadenosine, which cannot be phosphorylated to AMP, did not lead to inhibition of the glucagon-stimulated increase in glucose release as did adenosine.

Discrepancies between the activation state of glycogen phosphorylase and the rate of glycogen breakdown have been described. The catalytic efficiency of phosphorylase *a* as measured indirectly by the glycogenolytic capacity was shown to be decreased by  $\alpha$ -glucosidase inhibitors [32] and fructose [33,34], and to be increased by anoxia [35,36]. It has been suggested that the mechanisms involved are allosteric inhibition of phosphorylase *a* by fructose 1-phosphate and/or depletion of P<sub>1</sub> (fructose administration), and activation by an increased intracellular P<sub>1</sub> concentration (anoxia). Recent experiments using <sup>31</sup>P-n.m.r.

indicate that the rate of glycogenolysis catalysed by phosphorylase *a* depends linearly on the hepatic  $P_i$  concentration [36]. It is known that adenosine [37] and ATP [28] increase intracellular adenosine concentrations in isolated hepatocytes; it has also been demonstrated that adenosine decreases the cytosolic  $P_i$  concentration [38]. A tentative explanation for the inhibition of phosphorylase *a* described here could thus be a decrease in the availability of cytosolic  $P_i$  brought about by increased rephosphorylation of adenosine [39].

Potential of a weak adenosine response by transport inhibitors has been described for many tissues. This effect is generally explained by an increase in the availability of adenosine for extracellular receptor activation. However, one recent study describing adenosine effects on skin microcirculation suggested that uptake of adenosine reduces the  $A_2$  effect of exogenous adenosine [40]. In addition, it was shown recently that the adenosine-dependent release of histamine from basophils could be inhibited by methylxanthines and transport inhibitors, which is suggestive of both extracellular and intracellular nucleoside effects [41]. The potentiation of the stimulatory effects by NBTI described in this study cannot be explained by increased extracellular availability of adenosine, since the inhibitor did not change the adenosine concentrations during the experiment. Regulation of cell metabolism via uptake of adenosine may be a more general phenomenon. This hypothesis is strengthened by data showing that activation of glycolysis by adenosine in chicken erythrocytes proceeds without involvement of nucleoside binding to the membrane [42] and by the finding that adenosine is able to counteract the cyclic AMP-dependent induction of phosphoenolpyruvate carboxykinase in cultured hepatocytes [43].

From the data presented it is evident that ATP and adenosine qualitatively elicited the same metabolic responses: activation of phosphorylase, modest stimulation of glycogenolysis and antagonism of the hormone effect on glucose production. However, only ATP, and not adenosine, was capable of decreasing basal glucose output in cells cultured with 20 mM-glucose (Figs. 1 and 2); the reason for this is unknown. The two purines also differ in their dose-response curves (Fig. 3). It could be argued that the pronounced biphasic shape of the ATP curve reflects the inhibitory action of ATP-derived adenosine, which accumulates to high effective levels only at high initial external ATP concentrations. However, the weak effect of NBTI on the ATP effects points to the possibility that ATP may be transported directly across the membrane by a system different from the adenosine translocator [44]. Attempts to eliminate ATP-derived adenosine by adenosine deaminase failed; protein kinase A was still activated by ATP, although the bulk medium adenosine level was  $< 0.2 \mu\text{M}$ . Thus the difference in the dose-response curves presumably reflects a higher stimulatory potency of ATP due to activation of the  $P_2$  receptor (Fig. 5); this is also supported by the higher phosphorylase *a* level at  $10 \mu\text{M}$  agonist (Table 3). The stimulatory signal of ATP is mediated via the  $P_2$  receptor and, due to degradation of ATP to adenosine, also via the  $P_1$  receptor. This study also clearly demonstrates that the  $\beta$ -methylene-substituted ATP acts only through its degradation product adenosine in adult hepatocytes (Table 3).

The question must be raised as to whether the inhibitory signal seen in the isolated cell system is of physiological importance for the intact organ. Adenosine degradation occurs primarily in the non-parenchymal cells [45], and evidence has accumulated for a direct action of adenosine and ATP on the non-parenchymal cells [9,46]. Upon exposure to ATP or adenosine, these cells produce prostaglandins, which in turn stimulate glycogenolysis in hepatocytes [47-49]. From these data it seems questionable whether adenosine and ATP reach the hepatocyte at concentrations sufficient to elicit direct strong signals. Clearly, more

experiments are needed to unravel the physiological role of adenosine and ATP in the liver.

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