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Effects of adenosine, adenosine triphosphate and structural analogues on glucagon secretion from the perfused pancreas of rat *in vitro*

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1 The effects of adenosine, adenosine triphosphate (ATP) and structural analogues have been studied on glucagon secretion from the isolated perfused pancreas of the rat in the presence of glucose (2.8 mM).

2 Adenosine induced a transient increase of glucagon secretion. This effect was concentration-dependent in the range of 0.165 to 165 μM. ATP also induced an increase, but the effect was no greater at 165 μM than at 16.5 μM.

3 2-Chloroadenosine, an analogue more resistant to metabolism or uptake systems than adenosine, was more effective. Among the three structural analogues of ATP or ADP studied, β,γ-methylene ATP which can be hydrolyzed into AMP and adenosine had an effect similar to adenosine or ATP at the same concentrations (1.65 and 16.5 μM); in contrast α, β-methylene ATP and α, β-methylene ADP (resistant to hydrolysis into AMP and adenosine) were ineffective.

4 Theophylline (50 μM) a specific blocker of the adenosine receptor, suppressed the glucagon peak induced by adenosine, 2-chloroadenosine, ATP and β, γ-methylene ATP (1.65 μM).

5 An inhibitor of 5′nucleotidase, α,β-methylene ADP (16.5 μM), reduced the glucagon increase induced by ATP and did not affect the response to adenosine (1.65 μM).

6 These results support the hypothesis of adenosine receptors (P_1-purinoceptors) on the pancreatic glucagon secretory cells and indicate that ATP acts after hydrolysis to adenosine.

Introduction

Adenosine and adenosine triphosphate (ATP) have been shown to stimulate glucagon secretion from the isolated perfused pancreas of the rat (Weir *et al.*, 1975; Loubatières-Mariani *et al.*, 1976; 1982; Petrack *et al.*, 1981) and from the dog isolated pancreas perfused *in situ* (Bacher *et al.*, 1982). Adenosine and ATP are thought to act as neuromodulators in various organs and tissues, interacting with membrane bound receptors. Burnstock (1978) has proposed two types of purinoceptors: P_1 and P_2. Adenosine is a more potent agonist than ATP at the P_1-receptor, and methylxanthines are competitive antagonists, whereas the P_2-receptor can be stimulated by ATP and/or ADP. Previous work from this laboratory has led to the pharmacological characterization of a P_2-purinoceptor on B cells; the activation of this receptor stimulated insulin secretion (Loubatières-Mariani *et al.*, 1979, Chapal & Loubatières-Mariani, 1981).

The aim of this work was to investigate and characterize the purinoceptors on the A cell.

Methods

The experiments were carried out on the isolated perfused pancreas of the rat, according to the technique previously described (Loubatières *et al., 1969).* Male Wistar rats weighing 350 g and fed *ad libitum* were anaesthetized with sodium pentobarbitone 60 mg kg^{-1}, i.p. The pancreas was totally isolated from all neighbouring tissues and organs; it was perfused through its own arterial system with a Krebs-Ringer bicarbonate buffer containing bovine albumin (2 g l^{-1}). The Krebs buffer had the following ionic compositon (mM): NaCl 108, KH_2PO_4 1.19, KCl 4.74, $CaCl_2$ 2.54, $MgSO_4,7H_2O$ 1.19, $NaHCO_3$ 18 and glucose 2.8. A mixture of O_2 (95%) and CO_2 (5%) was bubbled through this medium (pH = 7.35). The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure the flow being about 2.4 ml min^{-1}. In all the experiments a 30 min adaptation period was allowed before taking the first sample for glucagon assay. A second sample was taken 15 min later. These two control samples were used to determine the secretion of glucagon

before stimulation. The Krebs solution supplemented with nucleosides or nucleotides was then perfused for 20 min. Samples were taken every min, throughout one min, during the first 6 min, then at 7, 10, 15 and 20 min. For each sample the flow rate was measured. An aliquot of 0.5 ml was immediately frozen in 50 μl of a mixture of EDTA (32 mM) and aprotinin (Zymofren: 10.000 UKI). In experiments with the antagonist or the 5'nucleotidase inhibitor, these were perfused for 5 min before and during the 20 min infusion of nucleotide. Glucagon was assayed in duplicate in the effluent from the pancreas by the radioimmunological method of Unger *et al.* (1970) using the 30 K antiserum for pancreatic glucagon. The coefficient of variation was 10% intra-assay and 15% interassay. The sensitivity, defined as the concentration of glucagon displacing 5% of the initially bound tracer, was 15 pg ml^{-1}. The glucagon output rate was obtained by multiplying the glucagon concentration (pg ml^{-1}) by the flow (ml min^{-1}).

Analysis of results

The kinetics of glucagon output rate were studied for each dose of adenosine, ATP and nucleoside or nucleotide analogues. The results for each point were calculated as a percentage of the starting value just before adding the agonist. The values obtained were 'normalized glucagon output rate'. Data are expressed as mean ± s.e. mean of *n* experiments.

In order to investigate if the increase in glucagon release to adenosine and ATP was concentration-related, we used the 'mean normalized glucagon output rate' over the first 6 min obtained as follows: AUC/6 (AUC = area under the curve of normalized glucagon output rate). The increase of 'mean normalized glucagon output rate' was then calculated in the following way:

$$\frac{\text{Average agonist AUC}}{6} - \frac{\text{average control AUC}}{6}$$

The values obtained were plotted as a function of the logarithm of adenosine or ATP concentration in the Krebs (0.165–165 μM).

The probability associated with differences between means were evaluated by Student's *t* test. The Student-Newman-Keuls test was used for multiple comparisons (Zar, 1974). Probability of 0.05 or less was considered singificant.

Drugs

Drugs used were: adenosine 5'-triphosphate (ATP) in the form of sodium salt and adenosine in crystallized form, from Boehringer Mannheim Corporation. α,β-methylene ATP as the lithium salt, β,γ -methylene ATP and α,β-methylene ADP as the sodium salt, and 2-chloroadenosine were obtained from Sigma Chemical Company. Theophylline was obtained from Rhône-Poulenc. Sodium pentobarbitone (Nembutal) from Clin Midy Laboratory. Aprotinin (Zymofren) from Specia Laboratory, EDTA in the form of disodium salt from Prolabo, Rhône-Poulenc.

Results

Effects of adenosine and ATP

Adenosine (1.65 to 165 μM) stimulated glucagon secretion from the rat isolated pancreas (Figure 1: on the graph the results are expressed as percentage of starting values, for each set of experiments the absolute values at 45 min (mean ± s.e.mean) are given in the legends). The hormone secretion which was declining in the presence of glucose 2.8 mM, immediate-

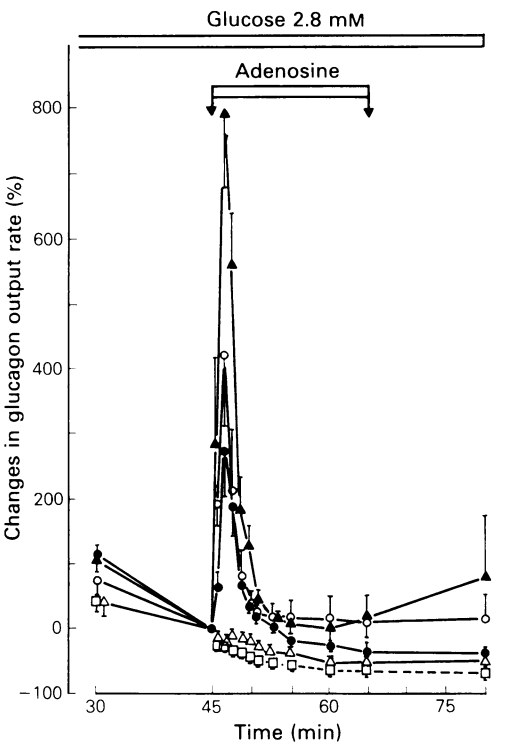

Figure 1 Effects of various concentrations of adenosine (0.165–165 μM) on glucagon secretion from the isolated perfused pancreas of rat. Each point represents the mean of 7 experiments; vertical bars show s.e.mean. Glucagon output rate (pg min^{-1}) at 45 min was for each set of experiments respectively: (▲) 165 μM: 587 ± 113; (o) 16.5 μM: 482 ± 81; (●) 1.65 μM: 432 ± 105; (△) 0.165 μM: 609 ± 96; (□) controls: 649 ± 71.

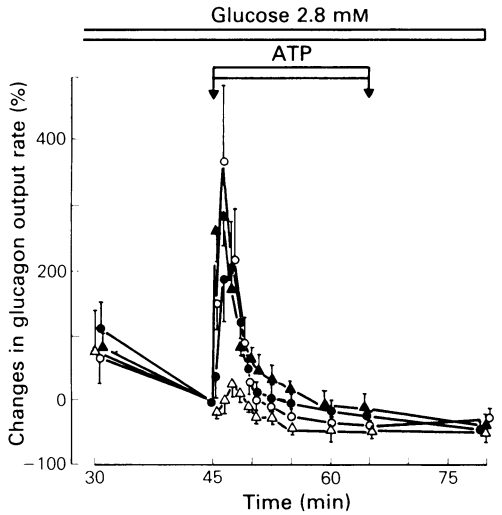

Figure 2 Effects of various concentrations of ATP (0.165–165 μM) on glucagon secretion from the isolated perfused pancreas of rat. Each point represents the mean, vertical bars show s.e.mean. Glucagon output rate (pg min^{-1}) at 45 min was for each set of experiments respectively: (▲) 165 μM: 443 ± 72 ($n = 10$); (o) 16.5 μM: 428 ± 91 ($n = 8$); (●) 1.65 μM: 430 ± 120 ($n = 5$); (△) 0.165 μM: 540 ± 171 ($n = 4$).

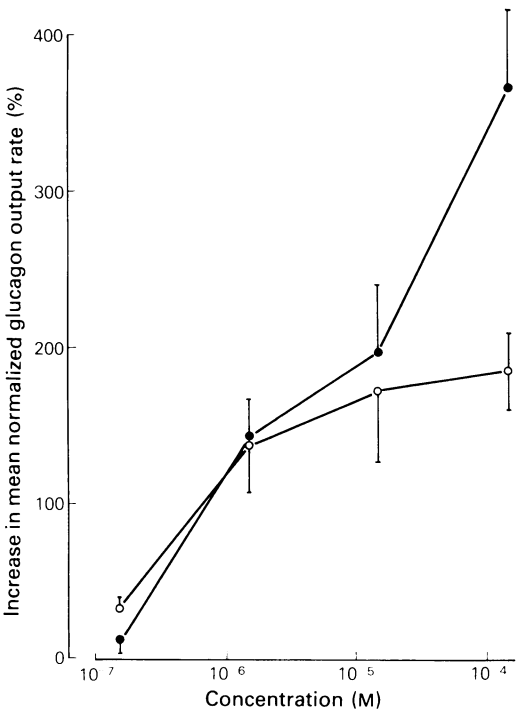

Figure 3 Increase of glucagon secretion induced by adenosine (●) and ATP (o). Each point represents the mean; vertical bars show s.e.mean).

ly increased in response to adenosine. The response culminated at the second min and lasted 6 min, although adenosine was continuously perfused for 20 min. The transient secretion of glucagon elicited by adenosine increased with the concentration.

ATP also increased glucagon secretion and the responses were similar to those obtained with adenosine at the concentrations of 0.165, 1.65 and 16.5 μM (Figure 2). In contrast ATP 165 μM did not induce a higher response than at 16.5 μM.

The difference between the responses to adenosine and ATP is illustrated by the concentration-response curves shown in Figure 3. The mean normalized glucagon output rates were not significantly different for the first three concentrations, but at the highest concentration (165 μM) the response to adenosine was significantly greater ($P < 0.05$) than that to ATP. The effect of adenosine was concentration-dependent in the range 0.165–165 μM.

Effects of structural analogues

Adenosine analogue 2-Chloroadenosine used at 1.65 μM induced a glucagon response with a pattern identical to adenosine (Figure 4), but the increase in glucagon secretion was significantly higher than that observed with adenosine at the same concentration ($P < 0.05$).

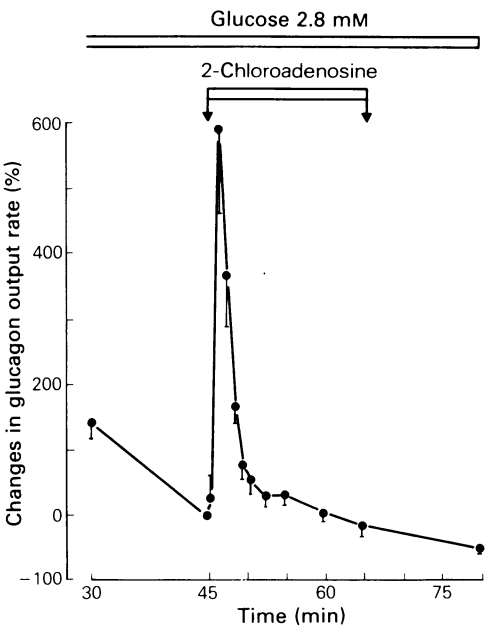

Figure 4 Effect of 2-chloroadenosine (1.65 μM) on glucagon secretion from the isolated perfused pancreas of rat. Each point represents the mean of 6 experiments; vertical bars show s.e. mean.

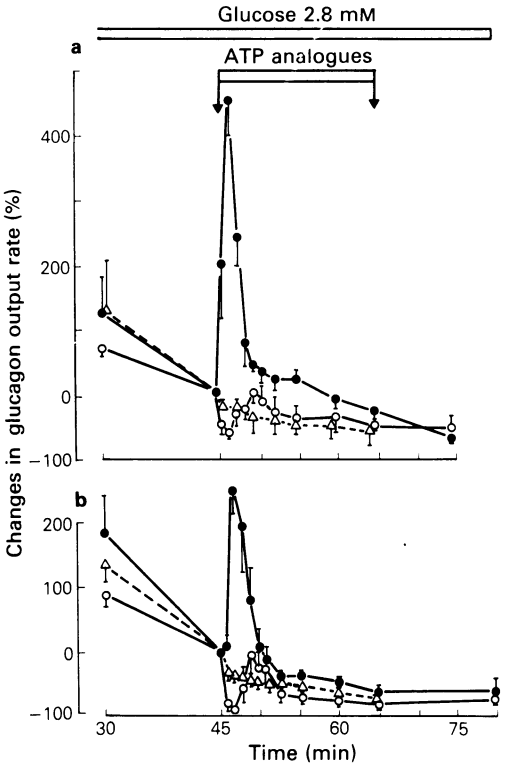

Figure 5 Effect of β,γ-methylene ATP (●) ($n=4$); α,β-methylene ATP (o) ($n=5$); α,β-methylene ADP (△) ($n=4$) at 16.5 μM (a) and 1.65 μM (b) on glucagon secretion from the isolated perfused pancreas of the rat. Each point represents the mean; vertical bars show s.e.mean.

ATP and ADP methylene analogues In these derivatives one oxygen atom of the di or triphosphate moiety is replaced by a methylene residue. The P-C-P bond is extremely stable and resistant to enzymatic cleavage. When the substitution was between P_β and P_γ, the ATP analogue induced a stimulation of glucagon secretion: β,γ-methylene ATP at 1.65 μM (Figure 5b) and 16.5 μM (Figure 5a) induced a glucagon response similar to that of adenosine or ATP at the same concentrations.

In contrast α,β-methylene ATP and α,β-methylene ADP which have the methylene substitution between P_α and P_β and cannot be metabolized to AMP and adenosine, were not active on glucagon secretion (Figure 5b: 1.65 μM and a: 16.5 μM). The brief inhibition seen with α,β-methylene ATP during the first minutes was due to a transient decrease in flow rate. This phenomenon was not observed with α,β-methylene ADP. The latter had no effect on the flow rate.

Experiments on antagonism: effect of theophylline

Theophylline was used at a concentration (50 μM) that had no effect on its own on glucagon secretion. When theophylline was infused 5 min before the agonist and throughout the next 20 min, it suppressed the glucagon peak induced by adenosine, ATP, 2-chloroadenosine and β,γ-methylene ATP (Table 1). The multiple comparison test showed that the glucagon secretion with these various treatments were not significantly different from the controls and from one another. Thus theophylline (50 μM) prevented the evoked release of glucagon by adenosine, ATP, 2-chloroadenosine and β,γ-methylene ATP.

Effect of an inhibitor of 5 nucleotidase: α,β-methylene ADP on ATP- and adenosine-induced glucagon secretion

α,β-methylene ADP (16.5 μM) was infused 5 min before and throughout the infusion of ATP or adenosine (1.65 μM) (Figure 6). The inhibitor of 5'nucleotidase had no effect on its own, as seen in

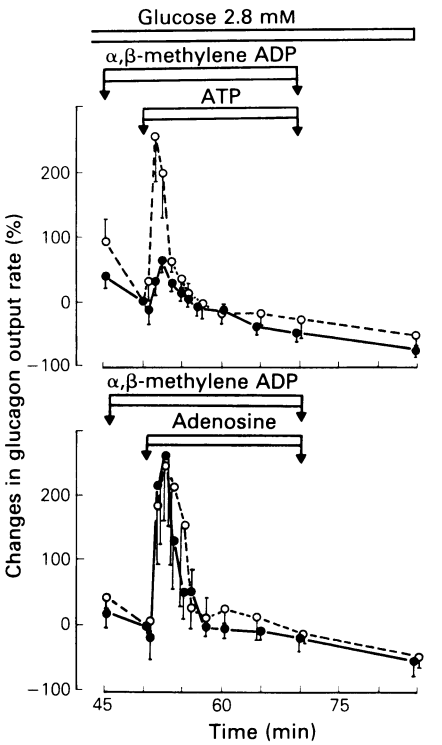

Figure 6 Effect of α,β-methylene ADP (16.5 μM) on glucagon secretion induced by ATP and adenosine at 1.65 μM. (o) ATP or adenosine alone; (●) ATP or adenosine in presence of α,β-methylene ADP. Each point represents the mean of 5 experiments; vertical bars show s.e.mean.

Table 1 Antagonism by theophylline of the glucagon secretion induced by adenosine, ATP, 2-chloroadenosine and β,γ-methylene ATP on the isolated perfused pancreas of the rat

		Minutes						
Drugs	n	1	2	3	4	5	6	10
Controls	5	87.8 ±8.3	86.8 ±11.5	83.2 ±5.8	83.2 ±8.4	80.0 ±6.5	81.8 ±8.7	81.8 ±12.6
Theophylline	8	91.0 ±10.8	91.6 ±18.0	87.5 ±14.5	82.0 ±13.7	81.6 ±13.0	76.8 ±14.2	72.0 ±15.2
Theophylline + adenosine	4	97.7 ±14.3	110.5 ±25.6	129.0 ±31.1	153.7 ±42.8	144.0 ±43.5	142.0 ±31.2	132.5 ±41.7
Theophylline + ATP	4	75.7 ±16.2	79.0 ±17.3	84.0 ±15.3	115.5 ±20.5	131.2 ±19.6	118.0 ±28.5	100.7 ±20.6
Theophylline + 2-chloroadenosine	4	90.2 ±3.3	93.7 ±7.1	106.0 ±3.9	140.2 ±22.7	132.0 ±40.7	117.7 ±22.5	97.0 ±12.1
Theophylline + β,γ-methylene ATP	4	123.2 ±39.3	98.7 ±8.3	104.0 ±12.1	109.5 ±14.6	99.5 ±8.9	89.0 ±7.1	91.2 ±31.2

The results are expressed as normalized glucagon output rate (as % of the value at 50 min just before the agonist infusion). Each value is the mean ±s.e.mean of the number of experiments (*n*). Theophylline (50 μM) was infused 5 min before the agonists (1.65 μM).

Figure 5, but the glucagon secretion induced by ATP was significantly reduced in the presence of α,β-methylene ADP ($P<0.01$). In contrast the effect of adenosine was not significantly modified.

Discussion

The present study shows that adenosine induces a transient increase of glucagon secretion in the presence of glucose 2.8 mM; this confirms previous results (Loubatières-Mariani *et al.*, 1982) and is in agreement with the studies of Weir *et al.* (1975) and Petrack *et al.* (1981) who also observed a stimulatory effect of adenosine on glucagon secretion, using the rat isolated pancreas perfused in the presence of glucose (4.2 mM). However, the adenosine concentration used by Weir *et al.* (1975) (2 mM) was much higher than that used in this study, whereas the single concentration (5 μM) tested by Petrack *et al.* (1981) was within the same range as our concentrations.

2-Chloroadenosine stimulated glucagon secretion and was even more effective than adenosine, though the effect was not of a longer duration, a result in agreement with Petrack *et al.* (1981). It is generally assumed that 2-chloroadenosine is resistant to adenosine deaminase (Clarke *et al.*, 1952) and not taken up by the cells (Muller & Paton, 1979; review by Daly, 1982). Consequently it can be concluded that the glucagon secretory effect of adenosine and 2-chloroadenosine is not dependent on intracellular metabolism but is due to an extracellular action. As the effect of these two substances is prevented by theophylline, a competitive antagonist of adenosine receptors at the concentration used (Okwuasaba *et al.*, 1977, Clanachan & Muller, 1980, Brown & Burnstock, 1981), the results suggest the presence of an adenosine receptor (P_1-purinoceptor) on the A cell.

In the present experiments, ATP also stimulated glucagon secretion; this is in contrast with the results obtained by Weir *et al.* (1975). However, it must be noted that these authors used different glucose and ATP concentrations and that their preparation included the duodenum. ATP is known to be rapidly broken down to adenosine. Since ecto-ATPases and 5′-nucleotidase hydrolyzing ATP to adenosine were described in islets of Langerhans (Lernmark *et al.*, 1976; Levin *et al.*, 1978) a rapid conversion of ATP to adenosine might occur in the vicinity of receptors. The fact that at a high concentration (165 μM) the effect of ATP was less than that of adenosine is consistent with ATP undergoing an enzymatic degradation before it is effective.

Among the structural analogues of ATP tested in the present work, the α,β-methylene isostere is resistant to hydrolysis to AMP and adenosine (Yount, 1975); it was ineffective on glucagon secretion, as it

was ineffective in the trachea (Christie & Satchell, 1980) and in cerebral cortical neurones (Phillis & Edstrom, 1976). In contrast, β,γ-methylene ATP can be metabolized to AMP and adenosine, as shown by Flodgaard & Torp-Pedersen (1978) who identified a calcium ion-dependent ATP pyrophosphohydrolase in plasma membrane from rat liver; this nucleotide analogue had a glucagon stimulatory effect, like ATP and adenosine. The β,γ-methylene isostere was also found to be an effective agonist on adenosine receptors by Phillis & Edstrom (1976) on cerebral cortical neurones, by Christie & Satchell (1980) in guinea-pig trachea and by McQueen & Ribeiro (1983) in the carotid body receptor of the cat. The finding that the stable ATP analogue (α,β-methylene ATP) was ineffective, and the hydrolysable ATP analogue (β,γ-methylene ATP) was effective, is consistent with ATP not acting directly, but through a hydrolysis product, probably adenosine.

In the presence of the 5'-nucleotidase inhibitor, α,β-methylene ADP, at a concentration which completely inhibited the activity of 5'-nucleotidase prepared from smooth muscle by Burger & Lowenstein (1970), the peak of glucagon secretion induced by ATP was significantly reduced, whereas the response to adenosine remained unchanged.

From our results, three arguments support the hypothesis that ATP acts after conversion into adenosine:(1) Among the methylene analogues used, β,γ-methylene ATP which can yield adenosine after hydrolysis of the bond between P_α and P_β, stimulated glucagon secretion. In contrast, α,β-methylene ATP and α,β-methylene ADP which cannot be converted to AMP and adenosine had no effect. (2) Theophylline, a specific competitive antagonist of adenosine, suppressed the increase in glucagon secretion induced by ATP and β,γ-methylene ATP. (3) α,β-methylene ADP which inhibits 5'-nucleotidase, thus preventing the production of adenosine from ATP, almost totally suppressed the glucagon secretion induced by ATP.

In conclusion our results support the hypothesis of adenosine receptors (P_1-type purinoceptors according to Burnstock's classification, 1978) on A cells, ATP being effective only after conversion to adenosine.

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