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Evidence for an A_2-subtype adenosine receptor on pancreatic glucagon secreting cells

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1 The effects of a 5'-substituted analogue of adenosine, 5'-N-ethylcarboxamidoadenosine (NECA) have been studied on glucagon secretion *in vitro*, using the isolated pancreas of the rat perfused in the presence of glucose (2.8 mM).

2 NECA provoked a peak of glucagon secretion, the kinetics of which were comparable to those previously obtained with adenosine. The effect was concentration-dependent and appeared at nanomolar concentrations. The EC_{50} was approximately 4×10^{-8} M.

3 A comparison of relative potency between adenosine and NECA showed that NECA was about 800 fold more potent than adenosine in inducing glucagon secretion.

4 Theophylline (50 μM) considerably decreased the peak of glucagon secretion induced by 1.65 μM NECA and totally suppressed the effect of 16.5 nM NECA. These results indicate the involvement of an adenosine receptor.

5 These and other previous results (low stereoselectivity of N^6-phenylisopropyladenosine) provide evidence for an adenosine receptor of the A_2-subtype being involved in glucagon secretion.

Introduction

Results of studies on the effects of adenosine and adenosine triphosphate (ATP) on pancreatic glucagon secreting cells have provided evidence for a P_1-purinoceptor on these cells (Loubatières-Mariani *et al.*, 1982). The activation of these receptors induced a transient increase of glucagon secretion. We have also shown that ATP was effective after hydrolysis to adenosine (Chapal *et al.*, 1984). It is now generally accepted that there are two subtypes of extracellular receptors involved in the action of adenosine. The A_1 subtype (according to Van Calker, 1979) or the Ri receptor (according to Londos *et al.*, 1980) with a high affinity for adenosine, is associated with inhibition of adenylate cyclase in many cell types (adipocytes, heart, brain cells). The other subtype (A_2 or Ra) has a lower affinity for adenosine and is often associated with a stimulation of adenylate cyclase. As it is not always possible to show that the effects of adenosine are linked to adenylate cyclase, it seems preferable to use the rank order of potencies of structural analogues to classify into A_1- or A_2-receptors (Fredholm, 1982; Collis & Brown, 1983). The most potent adenosine analogues at the A_1 adenosine receptor are the N^6-substituted compounds such as N^6-phenylisopropyladenosine (PIA) whereas at the A_2 adenosine receptor, 5'-substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA) are the most potent. In addition, the A_1 site exhibits marked stereospecificity, as L-PIA is 50 to 100 times more potent than its D-diastereoisomer (Brown & Collis, 1983) while the difference in potency between L- and D-PIA at the A_2 site is, at most, 5 fold (Smellie *et al.*, 1979; Collis & Brown, 1983; Burnstock *et al.*, 1984).

We have previously shown that L-PIA was not more potent than adenosine in stimulating glucagon secretion from the isolated perfused pancreas of the rat and that D-PIA was about 3 fold less potent than L-PIA (Loubatières-Mariani *et al.*, 1985). These results suggested that the receptor involved in glucagon secretion was not an A_1-receptor. In the present study we investigated the effects of NECA, a more specific A_2-agonist, and compared it with adenosine. We also studied the antagonism by theophylline of the effects of this structural adenosine analogue.

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 composition (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 95% O_2 and 5% CO_2 was bubbled through this medium (pH = 7.35). The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure (35 ± 5 cm, water) the flow being approx. 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 (at 45 min). These two control samples were used to determine the secretion of glucagon before adding the drugs to be tested. Each value for glucagon secretion is expressed as a percentage of the second sample at time 45 min. The Krebs solution supplemented with adenosine or structural analogue was then perfused for 20 min. Samples were taken every min, throughout 1 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 antagonist, this was perfused from time 45 min for 5 min prior to and during the 20 min infusion of agonist. Glucagon was assayed in duplicate in the effluent from the pancreas by the radioimmunological method of Unger *et al.* (1970) using the BR 124 antibody from the Institut de Biochimie Clinique, Geneva (Trimble *et al.*, 1982) and pork glucagon (Novo) as standard. 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 NECA and adenosine. The results for each point were calculated as a percentage of 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 whether the increase in glucagon release produced by adenosine and NECA was concentration-related, we used the 'mean normalized glucagon output rate' over the first 6 min which was 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 NECA concentration.

Drugs:

The following were used:- adenosine (Boehringer Mannheim Corporation), theophylline (Rhône-Poulenc), 5'-N-ethylcarboxamidoadenosine (kindly supplied by Byk Gulden Lomberg, Konstanz, FRG), sodium pentobarbitone (Nembutal, Clin Midy Laboratory), aprotinin (Zymofren, Specia Laboratory) and EDTA (Prolabo, Rhône-Poulenc).

Results

Effect of 5'-N-ethylcarboxamidoadenosine (NECA)

The 5' substituted analogue of adenosine (NECA) was

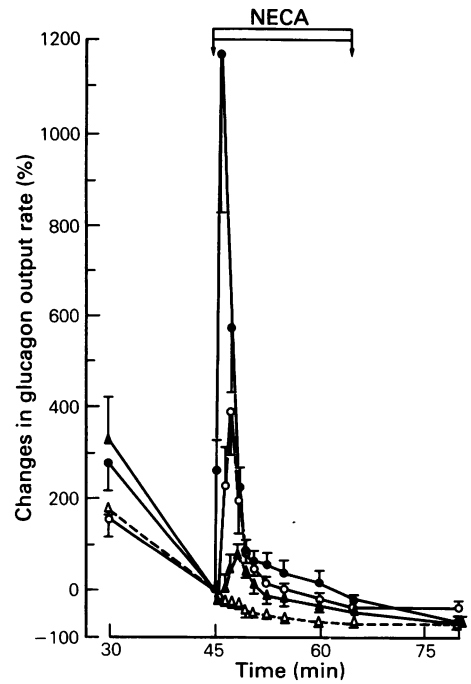

Figure 1 Effect of increasing concentrations of 5'-N-ethylcarboxamidoadenosine (NECA) on glucagon secretion from the isolated perfused pancreas of the rat: (▲) 3.3 nM ($n = 7$), (○) 16.5 nM ($n = 7$), (●) 165 nM ($n = 7$), (△) controls ($n = 9$). The glucagon output rate (pg min^{-1}) at 45 min for each set of experiments was 707 ± 74; 631 ± 210; 621 ± 88 and 691 ± 68 respectively. Each point represents the mean ± s.e.mean.

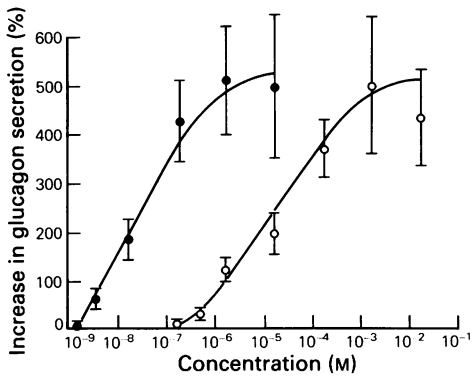

Figure 2 Increase in glucagon secretion, calculated as increase of 'mean normalized glucagon output rate' (see Methods), induced by (●) 5'-N-ethylcarboxamidoadenosine and (○) adenosine. Each point represents the mean with s.e.mean indicated by vertical lines. $n = 7$.

studied at concentrations ranging from 1.65 nM to 16.5 μM. In the range 3.3–165 nM NECA stimulated glucagon secretion in a dose-dependent manner (Figure 1). When NECA was added to physiological medium containing 2.8 mM glucose, the declining hormone secretion immediately increased. The maximal response occurred at the second min and lasted about 6 min. The time course of the response was similar to that previously observed with adenosine (Loubatières-Mariani *et al.*, 1982; Chapal *et al.*, 1984). In the present work our experiments with adenosine were completed by using three more concentrations, enabling a concentration-response curve to be constructed for concentrations ranging from 0.165 μM to 16.5 mM (Figure 2).

Comparison of relative potencies of NECA and adenosine

The concentration-response curves for NECA and adenosine are shown in Figure 2. A comparison of potency was performed by the parallel line assay method (Armitage, 1980). Linear regression analysis from the increase of mean normalized glucagon output rate was carried out. The two concentration-response curves did not deviate significantly from parallelism. NECA was approximately 800 fold more potent than adenosine. The EC_{50} of NECA was about 0.04 μM whereas the EC_{50} of adenosine was about 30 μM.

Antagonism by theophylline

Theophylline (50 μM) had no effect *per se* on glucagon secretion (Table 1); when it was infused 5 min before and throughout the next 20 min of NECA infusion (1.65 μM) it reduced considerably the glucagon secretion induced by the agonist. When using a concentration of NECA (16.5 nM) that induced about 35% of the maximum effect, the peak of glucagon secretion was totally suppressed by theophylline (Table 1).

Discussion

This study shows that the 5'-substituted analogue of adenosine (NECA) induces a transient and dose-dependent increase of glucagon secretion from the isolated perfused pancreas of the rat. These results obtained *in vitro* are in accordance with those obtained by Schütz *et al.* (1978a, 1979) *in vivo* in conscious dogs

Table 1 Antagonism by theophylline of glucagon secretion induced by 5'-N-ethylcarboxamidoadenosine (NECA), at two concentrations on the isolated perfused pancreas of the rat

		Minutes						*AUC*
Drugs	*n*	1	2	3	4	5	6	
Controls	8	86.5 ± 10.9	85.6 ± 7.1	76.0 ± 5.9	73.8 ± 8.0	78.4 ± 5.3	75.7 ± 6.6	476.1 ± 26.2
Theophylline	5	95.8 ± 4.6	89.2 ± 20.1	85.2 ± 13.5	78.5 ± 9.2	76.1 ± 11.0	71.0 ± 13.1	495.8 ± 64.8
NECA 1.65 μM	7	319.7 ± 129.8	1475.1 ± 399.4	813.6 ± 119.9	414.1 ± 60.6	267.4 ± 40.0	200.4 ± 31.1	3490.4 ± 631.7
NECA 1.65 μM + theophylline	4	81.4 ± 9.7	266.0 ± 66.4	395.0 ± 77.3	202.5 ± 45.8	163.0 ± 29.3	137.2 ± 15.1	1245.1 ± 222.3
NECA 16.5 nM	5	81.8 ± 8.5	217.8 ± 65.0	354.9 ± 105.3	180.1 ± 32.8	160.4 ± 16.1	131.6 ± 14.5	1126.6 ± 194.6
NECA 16.5 nM + theophylline	5	84.5 ± 11.7	92.6 ± 10.1	93.7 ± 15.3	86.2 ± 6.8	77.0 ± 10.3	74.8 ± 11.8	508.8 ± 60.9

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 NECA.

and in normal and reserpinized rats; but these authors were unable to demonstrate an effect of NECA *in vitro* on basal or arginine-induced glucagon release from isolated islets of Langerhans. According to these authors, this could be due to collagenase digestion in the isolation technique.

In the present work the peak of glucagon secretion induced by NECA on rat pancreas was suppressed by theophylline, as was the peak induced by adenosine in a previous study (Chapal *et al.*, 1984), indicating that NECA stimulates glucagon secretion by interaction with a cell surface adenosine receptor. These results are in agreement with those of Schütz *et al.* (1978b) who found that the increase in plasma glucagon levels after intravenous administration of NECA to dogs, was inhibited by pretreatment with aminophylline.

It is interesting to point out the high potency of NECA for inducing glucagon secretion, since it is effective at nanomolar concentrations. To our knowledge, such low concentrations of this adenosine analogue have never been shown to induce a physiological effect, either on adenylate cyclase or other biological systems, except however on glucagon release from the *in situ* isolated blood perfused pancreas of anaesthetized dogs (Bacher *et al.*, 1982). Since the 5'-substituted analogues of adenosine have such a high affinity for the receptor of the glucagon secreting cell, this receptor seems to be an adenosine receptor of the A_2-subtype, such as that described in other issues: platelets, liver, adrenal and Leydig cells (reviewed by Daly, 1982), guinea-pig trachea (Brown & Collis, 1982), rat brain microvessels (Schütz *et al.*, 1982), aorta (Collis & Brown, 1983) and guinea-pig taenia coli (Burnstock *et al.*, 1984).

We have previously studied the effect of N^6-substituted analogues of adenosine, N^6-phenylisopropyladenosine (D and L-PIA) on glucagon secretion (Loubatières-Mariani *et al.*, 1985). L-PIA had similar kinetics and activity to adenosine. D-PIA was 3 fold less potent than L-PIA. Thus, adenosine receptors of the glucagon secreting cell did not show a great stereoselectivity for PIA. This gave an additional support to identification of the A_2 subtype.

In conclusion, the present experiments provide evidence for an adenosine receptor of the A_2 subtype on the glucagon secreting pancreatic cells.

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