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Evidence for two different types of P2 receptors stimulating insulin secretion from pancreatic B cell

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1 Adenine nucleotides have been shown to stimulate insulin secretion by acting on P2 receptors of the P2Y type. Since there have been some discrepancies in the insulin response of different analogues of ATP and ADP, we investigated whether two different types of P2 receptors exist on pancreatic B cells. The effects of α,β-methylene ATP, which is more specific for the P2X subtype, were studied *in vitro* in pancreatic islets and isolated perfused pancreas from rats, in comparison with the potent P2Y receptor agonist ADPβS.

2 In isolated islets, incubated with a slightly stimulating glucose concentration (8.3 mM), α,β-me ATP (200 μM) and ADPβS (50 μM) similarly stimulated insulin secretion; by contrast, under a non stimulating glucose concentration (3 mM), α,β-me ATP was still effective whereas ADPβS was not. In the same way, in islets perifused with 3 mM glucose, α,β-me ATP but not ADPβS induced a partial but significant reduction in the peak ^{86}Rb efflux induced by the ATP-dependent potassium channel opener diazoxide.

3 In the isolated pancreas, perfused with a non stimulating glucose concentration (4.2 mM), ADPβS and α,β-me ATP (5–50 μM), administered for 10 min, induced an immediate, transient and concentration-dependent increase in the insulin secretion; their relative potency was not significantly different. In contrast, with a slightly stimulating glucose concentration (8.3 mM), ADPβS was previously shown to be 100 fold more potent than α,β-me ATP. Furthermore, at 4.2 mM glucose a second administration of α,β-me ATP was ineffective. In the same way, ADPβS was also able to desensitize its own insulin response. At 3 mM glucose, α,β-me ATP as well as ADPβS (50 μM) induced a transient stimulation of insulin secretion and down regulated the action of each other.

4 These results give evidence that pancreatic B cells, in addition to P2Y receptors, which potentiate glucose-induced insulin secretion, are provided with P2X receptors, which transiently stimulate insulin release at low non-stimulating glucose concentration and slightly affect the potassium conductance of the membrane.

Keywords: P2 receptors; insulin secretion; rubidium efflux; α,β-methylene ATP; ADPβS; desensitization

Introduction

Adenine nucleotides potentiate glucose-induced insulin secretion by acting on P2 receptors of the pancreatic B cell (Loubatières-Mariani *et al*., 1979; Chapal & Loubatières-Mariani, 1981a,b). These receptors have been pharmacologically characterized on the isolated perfused rat pancreas as being of the P2Y type (Bertrand *et al*., 1987), according to the classification by Burnstock & Kennedy (1985). This was further confirmed by experiments showing that the P2Y agonist adenosine-5′-O-(2-thiodiphosphate) (ADPβS) (Watson & Gilderstone, 1996), was about 100 fold more potent than ATP (Bertrand *et al*., 1991). The activation of these receptors elicited a strong and long lasting potentiation of the insulin secretion induced by a slightly stimulating glucose concentration. The pharmacological properties and physiological relevance of purinergic receptors of the pancreatic B cell have been reviewed elsewhere (Hillaire-Buys *et al*., 1994; Loubatières-Mariani *et al*., 1995; 1997; Petit *et al*., 1996). Recently, a cDNA clone encoding a rat P2Y receptor was isolated from an insulinoma cDNA library (Tokuyama *et al*., 1995). The stimulus-secretion coupling of B-cell P2 receptors is not yet definitely established and various mechanisms have been described. Both an increased influx of extracellular calcium (Petit *et al*., 1987; Geschwind *et al*., 1989) and an intracellular calcium mobilization (Gylfe & Hellman, 1987; Blachier & Malaisse, 1988) have been implicated. Furthermore, conflicting results have been reported regarding the involvement of polyphosphoinositide hydrolysis in the potentiating effect of adenine nucleotides on insulin release (Blachier & Malaisse, 1988; Petit *et al*., 1988). In addition, alterations in membrane potassium conductance by extracellular ATP or structural analogues have also been observed in mouse islets (Petit *et al*., 1989) and RINm5F insulinoma cells (Li *et al*., 1991). Hence if the presence of a P2Y receptor on insulin secreting B cells has been well documented, the discrepancies concerning the effects of different structural analogues of ATP and ADP on insulin release (Petit *et al*., 1989; Bertrand *et al*., 1989) and their mechanisms of action may be at least in part, ascribable to the existence of different types of P2 receptors on the B cells.

The aim of the present study was to investigate this hypothesis. For this purpose, we studied the effects of two stable P2 receptor agonists: α,β-methylene ATP and ADPβS, which are more specific for the P2X and the P2Y receptor, respectively (Watson & Gilderstone, 1996). These agonists were tested in different experimental conditions, particularly the glucose concentration in the medium, since we previously observed differences in the insulin response of isolated perfused rat pancreas to different agonists according to the ambient glucose concentration (Bertrand *et al*., 1989). Experiments were performed in two complementary types of preparations: isolated islets of Langerhans and isolated perfused pancreas. The former allowed us to test the effects of drugs both on

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insulin secretion and rubidium efflux as an indicator of potassium channel conductance. The latter (i.e. isolated pancreas) is of interest for the study of functional responses since the anatomical structure is preserved and the islets irrigated by their normal arterial system.

Methods

Experiments were performed *in vitro* in pancreatic islets or in isolated perfused pancreas from male Wistar albino rats fed *ad libitum* and weighing 300–350 g.

Experiments on isolated islets

Islets were isolated by collagenase digestion according to a technique derived from that of Lacy & Kostianowsky (1967).

In a first set of experiments, islets were stabilized during 90 min in a Krebs-Ringer bicarbonate buffer containing 8.3 mM glucose and supplemented with bovine serum albumine (Fraction V) 1 mg ml^{-1} and 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) 10 mM. Thereafter, batches of three islets were incubated for 30 min in 1 ml medium containing α,β-methylene ATP or ADPβS, in the presence of either 8.3 or 3 mM glucose. At the end of the incubation period, a sample of the medium was taken for insulin radioimmunoassay.

In a second set of experiments, islets were loaded for 90 min at 37°C with ^{86}Rubidium (37-185 Mbq ml^{-1}; specific activity 37–296 Mbq mg^{-1} rubidium), a tracer for potassium; batches of 25 islets were then perfused at a flow rate of 1.4 ml min^{-1} with the physiological solution containing glucose (3 mM) during 60 min (Petit *et al*., 1991). The ATP-dependent potassium channel opener diazoxide (100 μM) was added alone or simultaneously with α,β-methylene ATP or ADPβS, between min 40 and 60, and ^{86}Rb efflux was measured in each effluent fraction collected over 2-min intervals from min 20 onwards.

Experiments on isolated perfused pancreas

The pancreas was completely isolated according to a technique previously described (Loubatières *et al*., 1969) and perfused through its own arterial system with a Krebs-Ringer bicarbonate buffer containing 4.2 mM or 3 mM glucose and 2 g l^{-1} bovine serum albumin. A mixture of O_2 (95%) and CO_2 (5%) was bubbled through this medium at atmospheric pressure. The pH of the solution was 7.35. The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure (40–50 cm water) selected so as to produce a flow rate of 2.5 ml min^{-1} at the start of the experiment; in these conditions, any change in the flow rate reflects a change in vascular resistance (Hillaire-Buys *et al*., 1991). A 30 min adaptation period was allowed before the first sample was taken for insulin assay. Two samples were taken 10 and 15 min later, at time 40 and 45 min. Then, two successive infusions of α,β-methylene ATP or ADPβS were performed during 10 min and separated by a wash-out period of 20 min. In two sets of experiments the administration of drugs was crossed over, and so the administration of α,β-methylene ATP was followed by ADPβS administration and conversely. Insulin was measured in the effluent fluid.

The concentration-response curves for both drugs were established using the results obtained during the first administration of the drugs. The mean insulin output rate was calculated as follows: the area under the curve for the stimulating period divided by the number of minutes.

Assays

Insulin was assayed by the radioimmunological method of Herbert *et al*. (1965) using ^{125}I-insulin (C.I.S. International, Gif-Sur-Yvette, France), purified rat insulin as standard (Novo, Copenhagen, Denmark), the biological activity of which was 14.2 μU ng^{-1}, and anti-insulin serum (ICN biochemicals, Miles, Puteaux, France). The sensitivity of the assay was 0.1 ng ml^{-1}. Insulin release from incubated islets is expressed as ng/islet; insulin output from perfused pancreas is expressed as ng min^{-1} and was determined by multiplying the hormone concentration in the effluent fraction by the flow rate.

^{86}Rubidium in the effluent fraction and remaining in the islets at the end of the experiment was counted by measuring the Cerenkov radiation, after addition to each sample of 3 ml of a 3 mM aqueous solution of the wavelength shifter 7-amino-1,3-naphtalene-disulphonic acid, potassium salt (Henquin, 1978). From the sum of the radioactivity remaining in the islets at the end of the experiment and the cumulated effluent radioactivity, the fractional efflux of $^{86}Rb^{+}$ was calculated for each collection interval and expressed as a percentage of the instantaneous islet content per min. The peak $^{86}Rb^{+}$ efflux induced by diazoxide was calculated as the ratio of the highest value during diazoxide administration (between min 44 and 48) to the baseline efflux, between min 38 and 40 of perifusion.

Statistical analysis

Results are expressed as means $\pm$ standard error of the mean (s.e.mean). Data were submitted to analysis of variance and multiple comparison test of Newman-Keuls (Zar, 1974). Analysis of variance was also applied to the linear regression.

Chemicals

α,β-methylene ATP and diazoxide were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.); diazoxide was dissolved with an equimolar quantity of NaOH (1 mol l^{-1}) and the volume adjusted with NaCl (0.154 mol l^{-1}). Adenosine-5′-O-(2-thiodiphosphate) (ADPβS) was from Boehringer (Mannheim, Germany). Collagenase was supplied by Serva (Heidelberg, Germany), ^{86}RbCl by the Radiochemical Centre (Amersham, U.K.), HEPES by Gibco Europe (Paisley, U.K.), and bovine serum albumin (Fraction V) by the Sigma Chemical Co (St. Louis, MO, U.S.A.).

Results

Effects of α,β-methylene ATP and ADPβS on insulin secretion from rat isolated islets in the presence of either a slightly stimulating glucose concentration (8.3 mM) or a non stimulating glucose concentration (3 mM)

In a first set of experiments, the effect of ADPβS was investigated on isolated islets in the presence of 8.3 mM glucose. As shown in Figure 1, ADPβS increased insulin secretion from incubated islets; this effect is concentration-dependent and is significant from 50 μM. In the following experiments, the concentrations of ADPβS and α,β-methylene ATP, which was previously shown to stimulate insulin release from isolated islets (Petit *et al*., 1987), were chosen so as to obtain a similar increase in insulin secretion in the presence of 8.3 mM glucose. As seen on Figure 2, ADPβS at 50 μM and α,β-methylene ATP at 200 μM, stimulated insulin release in a similar way and increased it by about 40% ($P<0.01$). In

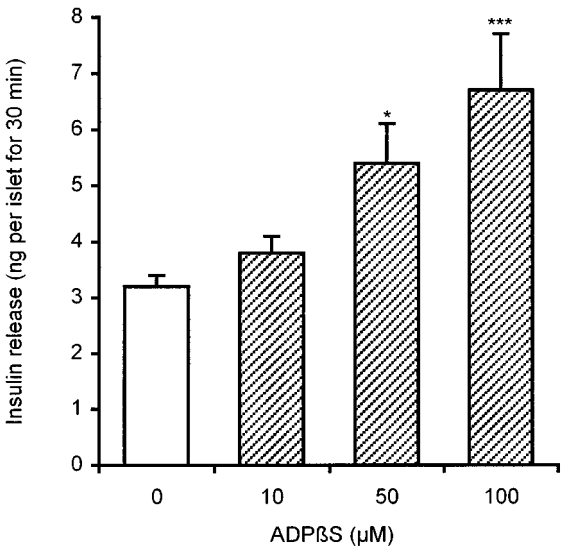

Figure 1 Effects of ADPβS on insulin release from rat isolated islets, incubated during 30 min in the presence of 8.3 mM glucose. The values are means$\pm$s.e.mean of 16–26 determinations obtained from 4–6 different experiments. *$P<0.05$ and ***$P<0.001$ versus controls.

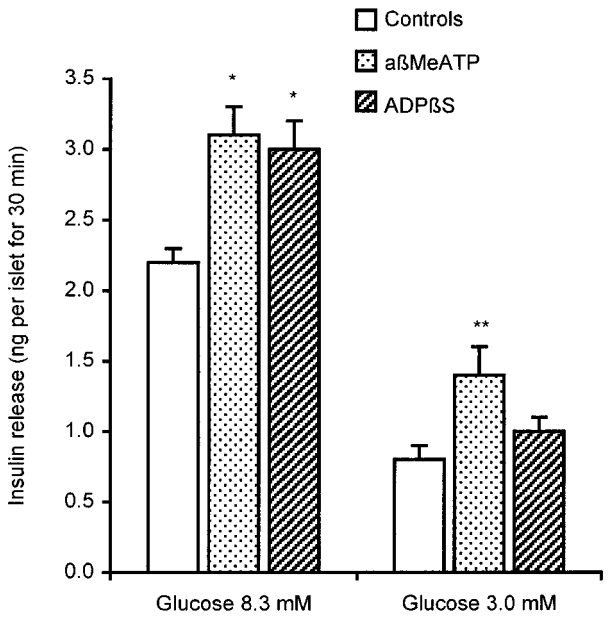

Figure 2 Effects of α,β-methylene ATP (200 μM) and ADPβS (50 μM) on insulin release from rat isolated islets, incubated during 30 min in the presence of 8.3 mM (left part) or 3 mM glucose (right part). The values are means$\pm$s.e.mean of 17–18 determinations obtained from 4–6 different experiments. *$P<0.05$ and **$P<0.01$ versus controls.

contrast, in the presence of 3 mM glucose only α,β-methylene ATP induced a significant increase in insulin secretion whereas ADPβS was ineffective. The insulin release with α,β-methylene ATP (200 μM) was 1.4 ± 0.2 ng per islet versus 0.8 ± 0.1 ng per islet in the presence of glucose alone ($P<0.005$) and versus 1.0 ± 0.1 ng per islet in the presence of 50 μM of ADPβS ($P<0.05$), this latter was not significantly different from the release with glucose alone.

Effects of α,β-methylene ATP and ADPβS on the ^{86}Rb efflux induced by the ATP-dependent potassium channel opener diazoxide (100 μM) in the presence of a non stimulating glucose concentration (3 mM)

In these experiments the basal fractional efflux of ^{86}Rb, which was $0.33\pm0.03\%$ of the instantaneous islet content per min, was immediately increased by diazoxide; the peak of ^{86}Rb efflux averaged $0.66\pm0.05\%$ per min, i.e. 2 fold the baseline efflux. The comparative effects of α,β-methylene ATP and ADPβS on the peak ^{86}Rb efflux induced by diazoxide are shown in Figure 3. When added simultaneously with diazoxide, α,β-methylene ATP induced a slight but significant reduction in the peak ^{86}Rb efflux, whereas ADPβS was ineffective. On this figure, it can be seen also the action of the hypoglycemic sulphonylurea tolbutamide (100 μM) which totally suppressed the diazoxide-induced increase in ^{86}Rb efflux.

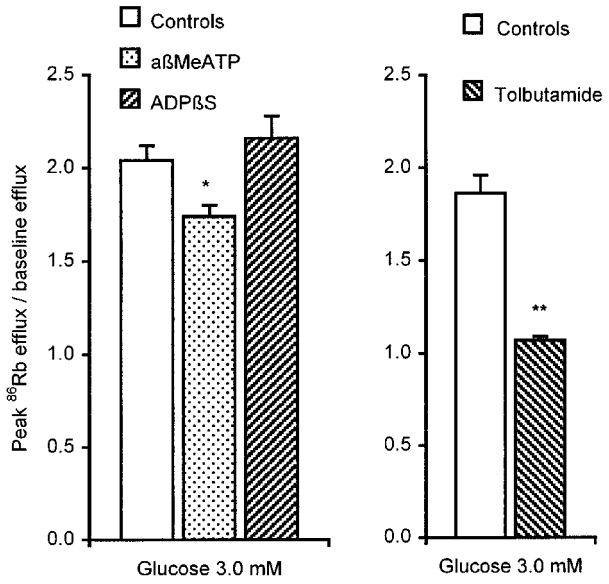

Figure 3 Left panel: effects of α,β-methylene ATP (200 μM) and ADPβS (50 μM) on the diazoxide-induced peak of ^{86}Rb efflux from perifused rat islets. Diazoxide and agonists are administered simultaneously (between min 40 and 60). The values are means $\pm$s.e.mean of 5–7 experiments. They express the ratio of the peak ^{86}Rb efflux (min 44–48), induced by diazoxide (100 μM) alone (controls) or with agonists to the baseline efflux just before addition of the drugs (min 38–40). *$P<0.025$ versus controls and ADPβS. Right panel: effects of tolbutamide (100 μM) on the diazoxide-induced peak of ^{86}Rb efflux; means$\pm$s.e.mean for three experiments. **$P<0.01$ versus controls.

Effects of α,β-methylene ATP and ADPβS on insulin secretion from the isolated perfused pancreas in the presence of a non stimulating glucose concentration

In the presence of 4.2 mM glucose both drugs were used in the range of 1.5–50 μM. The first application for 10 min of α,β-methylene ATP and ADPβS induced a transient increase in insulin release, which was in a peak form (Figure 4a and b); this effect was concentration-dependent with both drugs. In pancreas perfused with glucose alone, the insulin secretion remains stable. According to the experimental sets, insulin output ranged between 0.63 ± 0.18 and 1.23 ± 0.24 ng min^{-1}, just before the infusion of the agonist. The peak of insulin secretion induced by α,β-methylene ATP was slightly delayed (Figure 4a): it appeared after a brief (2 first min) decrease in insulin secretion, due to a drastic and transient reduction in the pancreatic flow rate, as previously described (Chapal & Loubatières-Mariani, 1983); this is not observed with ADPβS

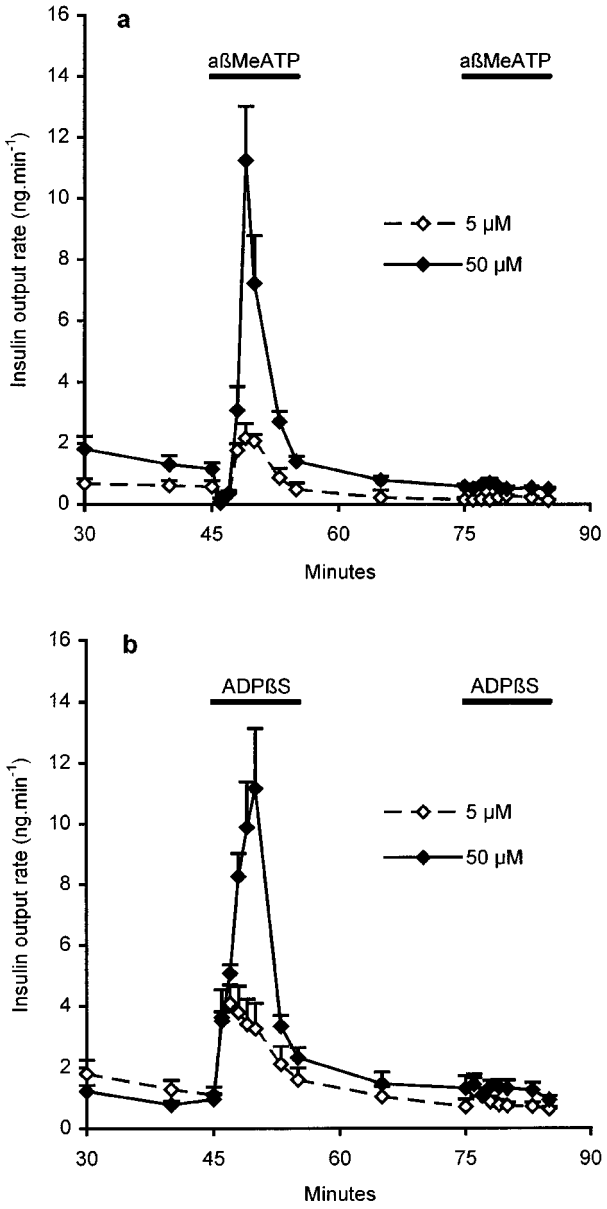

Figure 4 Effects of two successive administrations of α,β-methylene ATP (a) and ADPβS (b) on insulin secretion from the isolated perfused rat pancreas. Each point represents the mean and vertical lines s.e.mean of five experiments for α,β-methylene ATP and six experiments for ADPβS.

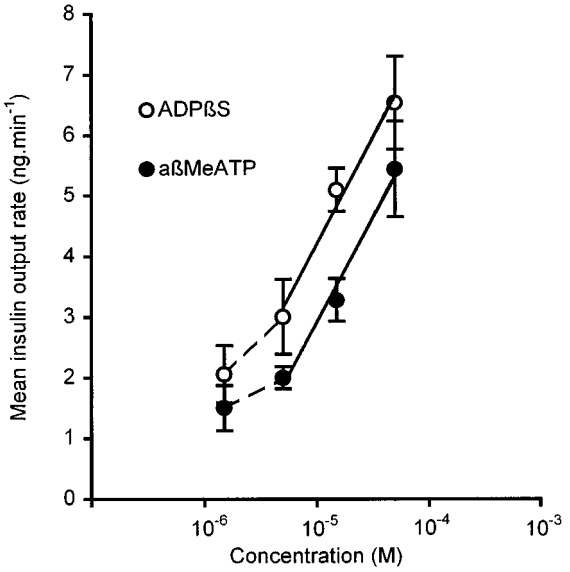

Figure 5 Concentration-response curves for α,β-methylene ATP and ADPβS-induced insulin secretion from the isolated perfused rat pancreas. Each point represents the mean and vertical lines s.e.mean of 5–7 experiments. Solid lines represent the calculated regression lines.

which is much less vasoconstrictor (Hillaire-Buys *et al*., 1998).

The concentration-response curves (Figure 5) were established using the mean insulin output rate as described in Methods. The comparison of the potency of α,β-methylene ATP and ADPβS was performed after analysis of variance of the regression, using the linear part of the curves. The two concentration-response curves did not differ significantly from linearity and did not deviate significantly from parallelism. The difference between the two agonists was not statistically significant.

Concerning the successive administration of the drugs (Figure 4a and b), it can be noted that during the wash out, the insulin secretion returned to baseline values. When α,β-methylene ATP as well as ADPβS were added for the second time, the insulin secretion was not modified, giving evidence for a desensitization of the receptor.

Furthermore, crossed desensitization experiments were performed in the presence of 3 mM glucose (a similar concentration as that used in isolated islet experiment) with α,β-methylene ATP and ADPβS at 50 μM (Figure 6). The baseline values of the mean insulin output rate before the first and the second administration of drugs ranged between 1.02 ± 0.33 and 0.50 ± 0.09 ng min^{-1}. The first administration of ADPβS or α,β-methylene ATP induced a similar stimulation of insulin secretion. In both types of experiments the second administration of one drug was significantly decreased in comparison to the first administration of the same or the other drug, supporting a crossed downregulation between the effects of ADPβS and α,β-methylene ATP.

Discussion

The present results in isolated islets further underline the crucial role of glucose in the insulin response to P2 agonists. Indeed, in isolated islets incubated in the presence of a slightly stimulating glucose concentration (8.3 mM), ADPβS as well as α,β-methylene ATP potentiate the secretion of insulin. This is in agreement with results previously obtained in the isolated perfused rat pancreas (Chapal & Loubatières-Mariani, 1981a; Bertrand *et al*., 1991). In contrast, when used at the same concentrations in the presence of a low, non stimulating glucose concentration (3 mM), only the more specific P2X agonist α,β-methylene ATP is able to enhance insulin secretion, the more specific P2Y agonist ADPβS being ineffective. These results suggest that the type of P2 receptor involved in the stimulation of insulin secretion in the presence of a low glucose concentration is different from that involved in the presence of a slightly stimulating glucose concentration.

Experiments on ^{86}Rb efflux were only performed, as usually, in the presence of 3 mM glucose since glucose, through its intracellular metabolism, interferes with plasma membrane potassium conductance and stimulates insulin secretion by closing ATP-dependent potassium channels (see Petit & Loubatières-Mariani, 1992). The administration of the ATP-

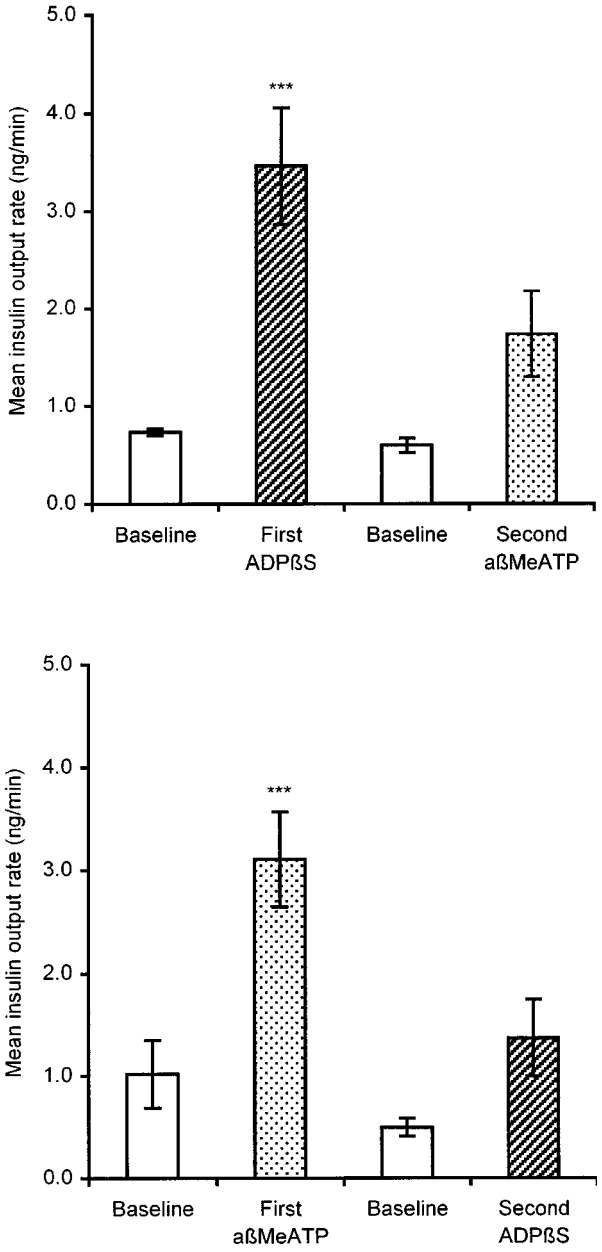

Figure 6 Effects of two successive administrations of ATP analogues (50 μM) on insulin secretion from the isolated perfused rat pancreas. In upper panel ADPβS was firstly administered and α,β-methylene ATP was added after the wash out period. In lower panel the drugs were administered in the reverse order. Baseline mean insulin output rate was calculated just before first and second administration of drugs for the period 40–45 min and the period 65–75 min respectively. The values are means$\pm$s.e.mean of six different experiments. ***$P<0.001$ versus baseline and the second administration of the same or the other drug.

dependent potassium channel opener diazoxide (Trube *et al*., 1986) is well known to rapidly increase the efflux of rubidium from the islets (Henquin & Meissner, 1982; Petit *et al*., 1991). It is interesting to note that only the agonist effective on insulin secretion at low glucose concentration, α,β-methylene ATP, decreases the peak of ^{86}Rb efflux induced by diazoxide, suggesting that this agonist may decrease the K^+ channel conductance in normal B-cell in these conditions. This is in agreement with previous results obtained by Li *et al*. (1991) on RINm5F insulinoma cells indicating that extracellular ATP doses ATP-dependent potassium channels. The slight reduction in the rubidium efflux may account, at least in part, for the insulin release induced by α,β-methylene ATP in the presence of 3 mM glucose. However it has to be underlined that this effect is weak in comparison to that of tolbutamide, which, in our conditions, completely suppressed the diazoxide-induced increase in Rb efflux.

Further evidence supporting that the pancreatic B cell is provided with two different types of P2 receptors is given by experiments performed in the isolated perfused pancreas. This preparation is more physiological than the isolated islets and presents several advantages. Indeed, the islets are continuously irrigated by their own normal capillaries, and the products of secretion are directly evacuated by the portal vein thus avoiding accumulation of other islet hormones (glucagon, somatostatin), which may influence insulin release, such accumulation is present in incubation procedure. It has also to be underlined that the isolated perfused pancreas is much more sensitive to ADPβS than the incubated islets. Indeed, in the present study, we observed that, in the presence of 8.3 mM glucose, 50 μM ADPβS was necessary to have a significant increase in the insulin release from incubated islets whereas in the isolated perfused pancreas, ADPβS began to be effective at 50 nM and had its maximal effect at 0.5 μM (Bertrand *et al*., 1991). Hence the concentrations used in isolated pancreas cannot be compared to that used in isolated islets.

In the presence of the non stimulating glucose concentration (4.2 mM) used for the isolated pancreas, the concentration-response curves of ADPβS and α,β-methylene ATP have a potency not significantly different whereas in the presence of 8.3 mM glucose previous studies have demonstrated that ADPβS was much more potent than α,β-methylene ATP; indeed ADPβS was 100 fold more potent than ATP (Bertrand *et al*., 1991), the potency of ATP being similar to that of α,β-methylene ATP (Chapal & Loubatières-Mariani, 1981a). Thus, ADPβS and α,β-methylene ATP show very different relative potencies in stimulating insulin secretion depending on the glucose concentration. These data support that different P2 receptors can be functionally evidenced at stimulating and non-stimulating glucose concentrations. In the presence of 8.3 mM glucose, as previously established, the P2 receptor is of the P2Y type; at low glucose concentration, the P2 receptor involved is more probably of the P2X type.

Another argument, well recognized for the pharmacological characterization of a P2X receptor is its desensitization (Kasakov & Burnstock, 1983; Kennedy, 1990). In the present work, a second administration of α,β-methylene ATP became ineffective, which supports the existence of a P2X receptor on insulin secreting B cell. Since we also observed a desensitization with ADPβS, we can propose that this analogue used at high concentrations can act on the B cell P2X receptor as was previously observed on pancreatic vessels (Hillaire-Buys *et al*., 1992; 1998). The crossed downregulation observed with α,β-methylene ATP and ADPβS (50 μM) in the presence of 3 mM glucose also supports the hypothesis that ADPβS, at the high concentrations used in these experiments, is no more specific for P2Y receptors and can act on P2X receptors. In support of the presence of a P2X type receptor on the pancreatic B cell, it was recently reported that a cDNA clone encoding a $P2X_4$ receptor was isolated from a rat pancreatic islet cDNA library (Wang *et al*., 1996); the $P2X_4$ mRNA was shown to be expressed in various hormone-secreting cell lines, including the insulin-secreting cell lines RINm5F and HIT-T15.

In conclusion, the present data show that two different types of P2 receptors stimulating insulin secretion can be functionally evidenced according to the ambient glucose concentration: a P2Y type at stimulating glucose concentration and a P2X

type at low, non-stimulating glucose concentration. From a physiological and pathophysiological point of view, this result is of interest because the P2X type receptor rapidly desensitizes itself, limiting the insulin-secreting response in the presence of a low glucose concentration thereby preventing a risk of hypoglycemia *in vivo*. As regards this risk, the wide margin between ADPβS concentrations needed to induce insulin secretion in the presence or absence of a stimulating glucose concentration, in contrast to the narrow margin for α,β-methylene ATP, is an argument for the development of P2Y selective agonists in the treatment of type 2 (non-insulin dependent) diabetes.

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