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ATP AND PHOSPHATE-MODIFIED ADENINE NUCLEOTIDE ANALOGUES

EFFECTS ON INSULIN SECRETION AND CALCIUM UPTAKE

P. PETIT, M. MANTEGHETTI, R. PUECH and M. M. LOUBATIERES-MARIANI*
Laboratoire de Pharmacologie, UA 599 du CNRS, Institut de Biologie, 34060 Montpellier Cédex, France

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Abstract—Our previous experiments on isolated rat pancreas gave evidence for a P_2 purinergic receptor on the insulin-secreting B cell. This work was designed to investigate whether the stimulation of insulin release by phosphorylated adenosine derivatives could also be observed in rat isolated Langerhans islets and whether this stimulation was accompanied by changes in calcium uptake. The results indicate that two structural methylene analogues of ATP and ADP (α,β-methylene ATP and α,β-methylene ADP) display an insulin stimulatory effect comparable to that of ATP, confirming the membrane action of the latter. It was also found that calcium uptake increased concomitantly with insulin release under the effect of α,β-methylene ADP; on the other hand this agent also increased the total exchangeable calcium content of islets at isotopic equilibrium. Verapamil, a blocker of voltage-sensitive calcium channels, counteracted the stimulation of insulin release and also blocked the increase in total exchangeable calcium content. These results demonstrate the involvement of calcium in the stimulus–secretion coupling of insulin release induced by an activator of P_2 purinergic receptors and suggest the implication of voltage-sensitive calcium channels.

ATP is known to play an essential intracellular role by acting as a fuel in the insulin secretion process [1, 2]. On the other hand, different pharmacological studies have provided evidence for a membrane receptor to ATP and/or ADP on the insulin-secreting B cell [3–5]. This purinergic receptor corresponds to the P_2 type, according to Burnstock's classification [6]. Stimulation of these receptors by ATP, ADP and some of their structural analogues in presence of a slightly stimulating glucose concentration (8.3 mM) results in a biphasic, concentration-dependent increase in insulin release from the isolated perfused rat pancreas [3].

This work was designed to investigate whether the stimulation of insulin release by phosphorylated adenosine derivatives could also be observed in isolated Langerhans islets, and whether this stimulation was accompanied with changes in calcium uptake by the islets. In these experiments we used ATP and stable structural analogues of ATP and ADP.

MATERIAL AND METHODS

Isolation of the islets. Pancreatic islets were isolated by the collagenase digestion technique [7] from male Wistar rats weighing 120–180 g. After washing, 100 islets were suspended in plastic Petri dishes containing 3 ml Eagle's minimal essential medium (MEM) with 10% heat inactivated calf serum, 20 mM sodium bicarbonate, 8.3 mM glucose, 400 IU/ml penicillin G and 200 μg/ml streptomycin sulfate. $CaCl_2$ concentration in culture medium was 1.8 mM. Batches of 100 islets were maintained in plastic Petri dishes during various times at 37°, pH 7.4 in an incubator gassed with O_2–CO_2 mixture (95%–5%).

Experimental protocols

Study of insulin release: Islets were maintained for 45 min in MEM containing 8.3 mM glucose. They were then transferred in groups of 10 into plastic tubes, and incubated for 5 min in 1 ml Krebs Ringer bicarbonate buffer containing HEPES 10 mM, bovine serum albumin (Fraction V) 2 g/l and glucose 8.3 mM. A 100-μl aliquot was sampled and the amount of insulin released during this period was measured.

During the following 5 min, islets were re-incubated either in presence of glucose alone 8.3 mM (controls) or in presence of ATP 16.5 μM or one of the following analogues: α,β-methylene ATP, α,β-methylene ADP, β,γ-methylene ATP each at 16.5 μM. A 100-μl sample was then assayed for insulin after the 10 min incubation period. The amount of insulin released in presence of the substance to be tested was obtained by calculating the difference between the two measurements.

Study of calcium movements. (i) Measurement of calcium uptake during insulin secretion: islets were maintained in the same conditions as above during a 45-min stabilization period. Each batch of 100 islets was then transferred into 3 ml MEM containing 8.3 mM glucose, $^{45}CaCl_2$ 10 μCi/ml, i.e. 0.37 MBq/ml (the specific radioactivity being approximately 5.5 μCi/mol or 0.2 MBq/mol), with α,β-methylene ADP, added into 100 μl saline (final concentration 165 μM). Control islets were also maintained in the same conditions and 100 μl saline were added into the Petri dishes.

* To whom correspondence should be addressed.

Insulin release and $[^{45}Ca]^{2+}$ content of the islets were measured at the end of a 30-min incubation period.

(ii) Measurement of calcium content at isotopic equilibrium: islets were incubated in plastic Petri dishes containing 3 ml MEM with 8.3 mM glucose and were labelled with $^{45}CaCl_2$ (0.37 MBq/ml, the final specific radioactivity being the same as already mentioned) for 24 hr to ascertain isotopic equilibrium. After 24 hr, α,β-methylene ADP in 100 μl saline solution, was added to the medium for 30 min (final concentration 16.5 μM). Control islets were performed by adding 100 μl saline into the medium. At the end of this incubation period, $[^{45}Ca]^{2+}$ content of the islets was measured.

In both experimental protocols, verapamil 50 μM was used as reference blocker of the voltage-sensitive calcium channels.

Measurements. Insulin was assayed by the radioimmunological B method of Hales and Randle [8] using the SB-INSI-1 kit from CEA (France). Purified rat insulin (kindly supplied by Novo, Copenhagen, Denmark) was used as reference standard, the biological activity of which was 19 μU/ng. Cross-reactions: Pro-insulin 7%, C-peptide 0.01%. The intra- and inter-assay coefficients of variations were respectively 9% and 13.5%. The results are expressed as ng per islet.

In order to assess the $[^{45}Ca]^{2+}$ content of the islets after each incubation, the 100 islets were transferred in groups of 10 into polyethylene microtubes, on top of an oil layer (dibutyl dinonyl phthalate 10:3 vol/vol) and centrifugated through the oil layer into 6 mol/l urea [9]. The bottom of the tube was cut above the urea layer and placed in 5 ml Instagel for liquid scintillation spectrometry. The $[^{45}Ca]^{2+}$ content of the islets was then calculated by subtracting the $[^{45}Ca]^{2+}$ contained in the extracellular space from the total $[^{45}Ca]^{2+}$. The extracellular space of islets, was measured in distinct groups of 10 islets (N = 17), using $[^{3}H]$sucrose. This extracellular space was 1.44 ± 0.15 nl/islet. The calcium uptake of islets was expressed as pmol/islet.

Chemicals. $^{45}CaCl_2$ and $[^{3}H]$sucrose were obtained from Radiochemical Centre (Amersham, U.K.). Collagenase was supplied by Serva (Heidelberg, F.R.G.). MEM and HEPES were from Gibco Europe (Paisley, Scotland). ATP, in the form of the sodium salt, was from Boehringer (Mannheim, F.R.G.). Albumin (Fraction V), α,β-methylene ATP as the lithium salt, α,β-methylene ADP and β,γ-methylene ATP in the form of the sodium salt, were from Sigma Chemical Company (St Louis, MO, U.S.A.). Instagel was from Packard Instrument (Rungis, France). Verapamil was kindly supplied by Biosedra (Malakoff, France).

Statistical analysis. The results are expressed as means ± SEM. Statistical comparisons were made either by using one-factor variance analysis and multiple comparison test [10] or Student's *t*-test.

RESULTS

Effects of ATP and structural analogues of adenine nucleotides on insulin secretion (Fig. 1)

ATP, α,β-methylene ADP and α,β-methylene ATP each at 16.5 μM significantly increased the amount of insulin released into the medium during the second period of 5-min incubation, respectively: 5.2 ± 1.1 vs 0.5 ± 0.4 ng/islet ($P < 0.001$); 5.0 ± 0.7 vs 0.4 ± 0.3 ng/islet ($P < 0.001$); 5.7 ± 1.5 vs 0.8 ± 0.3 ng/islet ($P < 0.001$).

β,γ-methylene ATP 16.5 μM did not induce any significant change in insulin secretion: 0.4 ± 0.3 vs 0.8 ± 0.5 ng/islet.

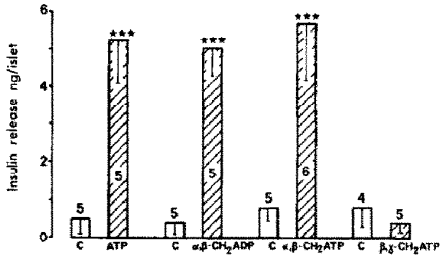

Fig. 1. Effects of ATP (16.5 μM), α,β-methylene ADP (16.5 μM), α,β-methylene ATP (16.5 μM) and β,γ-methylene ATP (16.5 μM) on insulin release by isolated rat islets of Langerhans incubated for 5 min in Krebs Ringer bicarbonate buffer (glucose 8.3 mM). C represents control islets. The number of experiments is indicated inside the columns. *** $P < 0.001$.

Effects of a stable analogue of ADP on calcium movements

In these experiments we used α,β-methylene ADP which is extremely stable and cannot be hydrolysed into AMP and adenosine.

Effects of α,β-methylene ADP on insulin secretion and calcium uptake (Fig. 2)

α,β-Methylene ADP 165 μM significantly increased the amount of insulin released by the islets during the 30 min incubation, as compared with the controls: 7.9 ± 0.8 vs 4.4 ± 1.0 ng/islet ($P < 0.02$). Calcium uptake was increased concomitantly: 7.8 ± 0.7 vs 5.5 ± 0.7 pmol/islet ($P < 0.02$).

In one experimental set we blocked the calcium channels with verapamil 50 μM. Under these conditions α,β-methylene ADP 165 μM failed to increase insulin release. The amount of insulin released (4.7 ± 0.3 ng/islet) was similar to that observed with control islets with or without verapamil (4.0 ± 0.5 ng/islet and 4.1 ± 0.8 ng/islet respectively) for three batches of 100 islets.

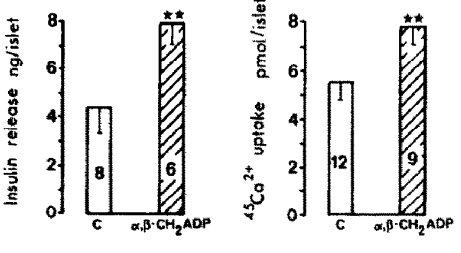

Fig. 2. Effects of α,β-methylene ADP (165 μM) on insulin release and $[^{45}Ca]^{2+}$ uptake by isolated rat islets of Langerhans incubated for 30 min in MEM (glucose 8.3 mM). C represents control islets. The number of experiments is indicated inside the columns. ** $P < 0.02$.

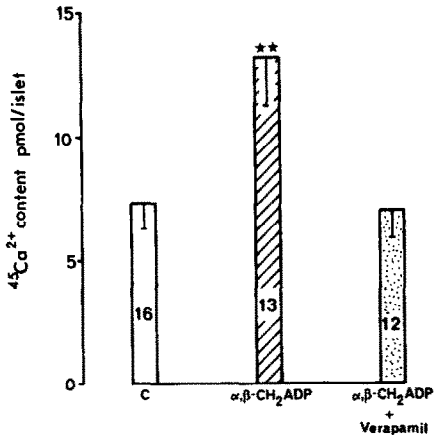

Fig. 3. Measurement of $[^{45}Ca]^{2+}$ content at isotopic equilibrium. Isolated rat islets of Langerhans were incubated for 30 min in MEM (glucose 8.3 mM) in presence of α,β-methylene ADP (16.5 μM) alone or with verapamil (50 μM). C represents control islets. The number of batches of 10 islets is indicated inside the columns. ** $P < 0.02$.

Effects of α,β-methylene ADP on calcium content (Fig. 3)

After 24-hr incubation, the addition of α,β-methylene ADP 16.5 μM for 30 min significantly increased the total exchangeable calcium content of the islets: 13.2 ± 1.9 vs 7.3 ± 1.0 pmol/islet ($P < 0.02$).

The adjunction of verapamil 50 μM inhibited the increase in calcium content observed with α,β-methylene ADP.

DISCUSSION

These results indicate that ATP induces an increase in insulin release from rat-isolated Langerhans islets. The structural methylene analogues of ATP and ADP are more resistant to degradation, due to stability of the P–C–P bonds which do not undergo enzymatic cleavage. The two structural analogues having a methylene residue between α and β phosphorus atoms (α,β-methylene ATP and α,β-methylene ADP) displayed an insulin stimulatory effect comparable to that of ATP, confirming the membrane action of the latter. In contrast, this effect was not observed with β,γ-methylene ATP, a structural analogue which possesses a methylene residue between β and γ phosphorus atoms. These results are in accordance with those previously obtained on the isolated perfused rat pancreas [4]. The difference in the response to the two types of analogues could be ascribed to structural and electronic characteristics [11, 12] of the polyphosphate chain, as discussed previously [4].

α,β-Methylene ADP, a stable analogue as or more potent than ATP [4] was chosen for our subsequent experiments, in order to exclude the desensitization phenomenon occurring with α,β-methylene ATP, as reported for example by Hedlund *et al.* [13], Sneddon and Burnstock [14], and Kennedy *et al.* [15].

It is well established that calcium plays an essential role in the stimulus–secretion coupling; this is the case for insulin secretion induced by glucose [16], sulphonylureas [17–20] or HB 699, a hypoglycaemic agent without the sulphonylurea group [21]. The present work shows that calcium uptake increases concomitantly with insulin release under the effect of α,β-methylene ADP. Moreover, during an identical incubation time, α,β-methylene ADP increases the total exchangeable calcium content in islets, preincubated with $[^{45}Ca]^{2+}$ for 24 hr under tissue culture conditions.

Verapamil, the reference blocker of calcium channels, has been used in various tissues to establish whether calcium uptake was mediated by voltage-sensitive or insensitive channels [22, 23] and also to discriminate between both processes in the Langerhans islets where the two types of channels may coexist [24]. At the concentrations used in this work and under conditions of isotopic equilibrium, verapamil did not display any effect *per se* on insulin secretion and islet calcium content [21]. In the same way, verapamil by itself did not affect insulin release (as indicated in the results), nor calcium uptake (unpublished results) under our experimental conditions with freshly isolated islets. In contrast, verapamil counteracted the increase in insulin release elicited by α,β-methylene ADP, and blocked as well the increase in total exchangeable calcium content at isotopic equilibrium and during the same incubation time.

These results demonstrate the involvement of calcium in the stimulus–secretion coupling of insulin release induced by α,β-methylene ADP, a stable ATP analogue. Like most secretagogues, it requires the accumulation of calcium for insulin secretion to be stimulated. Unlike arginine which would essentially involve the participation of voltage-insensitive channels [25], but like tolbutamide [19, 20] and HB 699 [21], the increase in calcium uptake necessary to stimulation of insulin secretion by activators of P_2 purinergic receptors seems to occur via voltage-sensitive calcium channels.

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