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DIFFERENTIAL EFFECTS OF PURINERGIC AND CHOLINERGIC ACTIVATION ON THE HYDROLYSIS OF MEMBRANE POLYPHOSPHOINOSITIDES IN RAT PANCREATIC ISLETS

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Abstract—This work was designed to investigate the effects of a P_2 purinoreceptor agonist, α,β-methylene ADP, on membrane polyphosphoinositide hydrolysis in relation to insulin release from rat isolated islets of Langerhans. The effects of this stable structural analogue of ADP (10^{-4} M) were compared with those of a muscarinic cholinergic agonist, carbachol (10^{-4} M). The interaction between α,β-methylene ADP and carbachol was studied on polyphosphoinositide breakdown and insulin secretion. The experiments were performed in presence of a slightly stimulating glucose concentration (8.3 mM). Whereas carbachol-induced insulin release was accompanied by a concomitant increase in inositol phosphates accumulation, α,β-methylene ADP at the same concentration produced a similar insulin secretion without eliciting an accumulation of inositol phosphates. The combined effect of both substances added simultaneously resulted in a significant increase in insulin release as compared with the secretion induced by either substance used separately. By contrast, the accumulation of inositol phosphates induced by both substances was not different from the accumulation induced by carbachol alone. These results seem to rule out the involvement of polyphosphoinositide hydrolysis in the coupling mechanism between P_2 purinoreceptor activation and insulin response of the B cell. Moreover, purinergic stimulation appears not to interact with the effect of muscarinic stimulation on polyphosphoinositide breakdown.

The existence of P_2 purinergic receptors on the insulin-secreting B cell has been well established and pharmacologically characterized [1–3]. Stimulation of these receptors by ATP, ADP and some of their structural analogues in presence of a slightly stimulating glucose concentration results in a biphasic, concentration-dependent increase in insulin release from the isolated perfused rat pancreas [1, 2].

The coupling mechanism between activation of the P_2 purinergic receptors of the B cells and insulin secretion is not clearly understood. Calcium appears to be involved in this functional coupling. We have shown that purinergic agonists increased $^{45}Ca^{2+}$ uptake in isolated islets [4]. On the other hand, Hellman [5] observed a purine nucleotide initiation of $^{45}Ca^{2+}$ efflux from pre-loaded islets and suggested that P_2 purinoreceptors mediate a similar mobilization of calcium as noted when activating polyphosphoinositide breakdown with carbachol.

This work was designed to investigate the effects of a P_2 purinoceptor agonist on membrane polyphospoinositide hydrolysis in relation with insulin release from rat isolated islets of Langerhans. To this end, we used a stable structural analogue, α,β-methylene ADP, which cannot be hydrolysed into AMP and adenosine, and which was shown to stimulate insulin secretion and $^{45}Ca^{2+}$ uptake in our experimental system [4]. We compared the effects of this purinergic agonist with those of a muscarinic cholinergic agonist, carbachol. In addition, we investigated the effects of α,β-methylene ADP and carbachol, used separately or in combination, on polyphosphoinositide breakdown and insulin secretion.

MATERIALS AND METHODS

Isolation of the islets. Our experiments were performed on pancreatic islets from male Wistar rats fed *ad libitum* and weighing 120–180 g. Islets were isolated by collagenase digestion [6] according to a technique derived from that of Lacy and Kostianowsky [7].

Incubation of the islets. Groups of 100 islets were suspended in 250 μl Krebs–Ringer bicarbonate buffer (pH 7.4) containing hydroxyethylpiperazineethanesulphonic acid (HEPES) 10 mM, bovine serum albumin (Fraction V) 2 g/l and glucose 8.3 mM. In these conditions, under continuous gassing with 95% O_2 plus 5% CO_2, islets were preincubated for 2 hr at 37° in a shaking incubator, in presence of 2-[^{3}H]myoinositol 10 μCi, i.e. 0.37 MBq (specific activity: 17.1 Ci/mmol, i.e. 0.6 TBq/mmol).

At the end of this labelling period, the radioactive preincubation medium was removed and the islets were washed twice with 500 μl fresh buffer containing no radioisotope.

Each group of 100 islets was then incubated in 1 ml of Krebs–Ringer bicarbonate buffer containing HEPES 10 mM, bovine serum albumin (Fraction V) 2 g/l, glucose 8.3 mM, lithium chloride 10 mM and myo-inositol 1 mM during a first phase of 10 min. A

100 μl aliquot was sampled and the amount of insulin released during this period was measured; 100 μl of the same medium were promptly added without (controls) or with the test substances, carbachol and/or α,β-methylene ADP, each at the final concentration of 10^{-4} M. The islets were further incubated in the same conditions during a second phase of 20 min. At the end of this period a 100-μl aliquot was sampled in order to measure the amount of insulin released during the 30 min incubation. The amount of insulin released in presence or not of the test substance was obtained by calculating the difference between the two values.

The incubations were terminated by the addition of 3 ml $CHCl_3/CH_3OH$/concentrated HCl (200:100:1, vol./vol.) and 100 μl EDTA 100 mM [8]. The tubes were vortex-mixed for 30 sec and then centrifuged for 15 min at 1500 *g* to separate the two phases.

Assays of inositol phosphates. Free inositol and inositol phosphates were eluted by anion exchange chromatography [9].

The upper aqueous phase was neutralized with 150 μl NaOH 1 N, diluted with 2.5 ml H_2O and applied to columns containing 1.5 ml Dowex 1 × 8, 100–200 mesh, formate form (Fluka AG, Buchs, Switzerland).

Free [^{3}H]inositol was eliminated by 12 ml H_2O, [^{3}H]glycerophosphoinositol was eluted with 10 ml of 5 mM disodium tetraborate, 60 mM sodium formate. Inositol phosphates were then eluted by stepwise addition of ammonium formate solutions of increasing ionic strength: IP with 12 ml NH_4 COOH 0.2 M, HCOOH 0.1 M; IP_2 with 16 ml NH_4 COOH 0.4 M, HCOOH 0.1 M; IP_3 with 16 ml NH_4 COOH 1 M, HCOOH 0.1 M.

According to some recent publications, IP and IP_2 fractions may not only contain inositol 1-monophosphate and inositol 4,5-bisphosphate respectively, but perhaps also their cyclic forms [10]. Likewise, the IP_3 fraction may consist of two isomers, inositol 1,4,5- and 1,3,4-trisphosphate [11]. It is clear that the anion exchange chromatographic method is not suitable for the analysis of these different inositol phosphate fractions.

Ten millilitres of liquid scintillation cocktail (ACS II, Amersham Corporation, Arlington Heights, U.S.A.) were then added to 8 ml of each eluate. The total radioactivity in each fraction was determined by scintillation counting and corrections for quenching of ^{3}H label were made by a quench curve based on the external standard ratio.

Extraction of lipids. The lower organic phase was dried under nitrogen stream, and the particulate material extracted with 3 ml $CHCl_3/CH_3OH$/concentrated HCl (200:100:1, vol./vol.). The extract was washed once with 1 ml H_2O, and once with 1 ml CH_3OH/H_2O (1:2 vol./vol.) [8]. The extract was again evaporated and redissolved in 20 ml scintillation fluid. The radioactivity of the [^{3}H] phosphoinositides was counted in a liquid scintillation spectrometer.

Insulin assay. Insulin was assayed by the radioimmunological B method of Hales and Randle [12] using the SB-INSI-1 kit from CEA (Commissariat à l'Energie Atomique, France). The standard used was purified rat insulin (Novo, Copenhagen, Denmark) the biological activity of which was 22.3 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%.

Result analysis. The radioactivity of the inositol-containing lipids as well as that of each hydrosoluble ester phosphate was calculated in dpm. According to our labelling conditions, incorporation of labelled inositol into the lipids averaged 74,752 ± 13,838 dpm/100 islets in the control experiments (N = 10). No significant difference was seen between this value and the values observed in the treated islets. Polyphosphoinositide hydrolysis, per group of 100 islets, is expressed by the radioactivity of the inositol phosphates in percent of the total radioactivity counted both in the inositol phospholipids and inositol phosphates.

Insulin secretion (in ng/100 islets) during the 20 min incubation in the presence of the agonists was calculated by the difference between the 30 and 10 min values, after correction for the dilution factor due to the addition of 100 μl medium after the first 10 min.

The results are expressed as means ± SEM. Statistical comparisons were made by using one-factor variance analysis and multiple comparison test [13].

Chemicals. Collagenase was supplied by Serva (Heidelberg, F.R.G.). 2-[^{3}H]myo-inositol was from New England Nuclear, (Boston, MA), HEPES from Gibco Europe (Paisley, U.K.). Carbachol (carbamylcholine chloride), α,β-methylene ADP in the form of the sodium salt, and albumin were from Sigma Chemical Company (St Louis, MO).

RESULTS

1. *Effects of α,β-methylene ADP and carbachol on insulin release and inositol phosphates accumulation (Fig. 1)*

α,β-methylene ADP and carbachol, each at 10^{-4} M, induced a similar and significant increase in insulin release during the second 20 min incubation period, respectively: 23.9 ± 4.5 (N = 10) and 24.4 ± 5.4 (N = 6) vs 10.2 ± 2.1 (N = 10) ng/100 islets ($P < 0.05$).

While carbachol concomitantly increased the accumulation of inositol phosphates, 38.4 ± 2.9% (N = 6) vs 22.3 ± 1.9% (N = 10) ($P < 0.001$), α,β-methylene ADP did not increase this accumulation: 18.8 ± 1.8% (N = 10) vs 22.3 ± 1.9% (N = 10) (NS). The values for each inositol phosphate fraction in these different incubation conditions are indicated in Table 1.

2. *Effects of α,β-methylene ADP and carbachol, added separately or in combination, on insulin release and inositol phosphates accumulation (Fig. 2)*

α,β-methylene ADP and carbachol, each at 10^{-4} M, added simultaneously to the incubation medium, elicited a significant increase in insulin release as compared with the secretion induced by either substance used separately: 43.5 ± 7.5 (N = 8) vs respectively 24.5 ± 5.5 (N = 11) ($P < 0.05$) and 21.4 ± 3.8 (N = 8) ng/100 islets ($P < 0.05$).

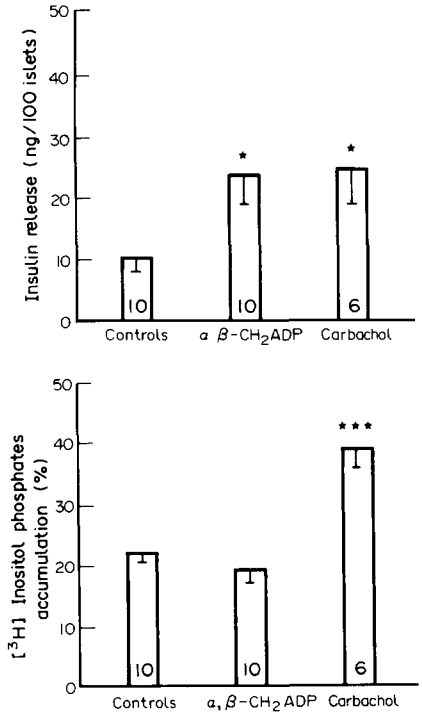

Fig. 1. Effects of α,β-methylene ADP (10^{-4} M) and carbachol (10^{-4} M) on insulin release and inositol phosphates accumulation by rat isolated islets of Langerhans incubated in a Krebs–Ringer bicarbonate buffer (10 mM LiCl, 8.3 mM glucose) and stimulated for 20 min. Each determination was performed with 100 islets. Inositol phosphates accumulation is expressed in percent of the total radioactivity counted both in the inositol phospholipids and inositol phosphates. Results are means ± SEM for the number of determinations indicated inside the columns. * $P < 0.05$; *** $P < 0.001$.

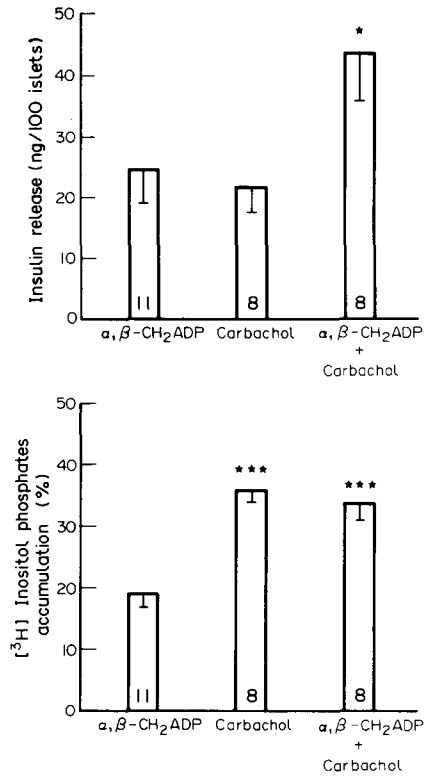

Fig. 2. Effects on insulin release and inositol phosphates accumulation of α,β-methylene ADP (10^{-4} M) and carbachol (10^{-4} M) added separately or in combination for 20 min. Isolated rat islets of Langerhans were incubated in a Krebs–Ringer bicarbonate buffer (10 mM LiCl, 8.3 mM glucose). Each determination was performed with 100 islets. Inositol phosphates accumulation is expressed in percent of the total radioactivity counted both in the inositol phospholipids and inositol phosphates. Results are means ± SEM for the number of determinations indicated inside the columns. * $P < 0.05$; *** $P < 0.001$.

By contrast, the accumulation of inositol phosphates induced by α,β-methylene ADP (10^{-4} M) and carbachol (10^{-4} M) used in combination, was not significantly different from the accumulation induced by carbachol alone at the same concentration: 33.6 ± 2.4% (N = 8) vs 36.1 ± 2.0% (N = 8). The values for each inositol phosphate fraction in these different conditions are indicated in Table 2.

DISCUSSION

The stimulation of insulin release induced by carbachol, an agonist of the muscarinic cholinergic receptors, is accompanied by an increase of inositol phosphates accumulation in the islets of Langerhans.

Table 1. Radioactivity of each inositol phosphate fraction in the different incubation conditions: controls, α,β-methylene ADP (10^{-4} M) and carbachol (10^{-4} M)

Incubation conditions	Inositol phosphate-associated radioactivity (dpm/100 islets)		
	Inositol phosphate	Inositol bisphosphate	Inositol trisphosphate
Controls (N = 10)	13657 ± 1353	3806 ± 624	2248 ± 285
α,β-Methylene ADP (N = 10)	13208 ± 1935	2692 ± 385	2050 ± 307
Carbachol (N = 6)	44633 ± 6810***	5458 ± 742*	4005 ± 583**

The inositol phosphates were separated by anion exchange chromatography. Each value is the mean ± SEM for the number of determinations indicated in parentheses, each determination being performed with 100 islets. Levels of significance relative to control islets: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Table 2. Radioactivity of each inositol phosphate fraction in the different incubation conditions: α,β-methylene ADP (10^{-4} M), carbachol (10^{-4} M) used separately or in combination

Incubation conditions	Inositol phosphate-associated radioactivity (dpm/100 islets)		
	Inositol phosphate	Inositol bisphosphate	Inositol trisphosphate
α,β-Methylene ADP (N = 11)	16349 ± 2434	3314 ± 448	2659 ± 331
Carbachol (N = 8)	44173 ± 5575***	5540 ± 627*	4202 ± 468*
α,β-Methylene ADP + carbachol (N = 8)	38322 ± 3934***	5921 ± 713**	3634 ± 422

The inositol phosphates were separated by anion exchange chromatography. Each value is the mean ± SEM for the number of determinations indicated in parentheses, each determination being performed with 100 islets. Levels of significance relative to the values for α,β-methylene ADP alone: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

The coupling of muscarinic receptor activation to hydrolysis of membrane polyphosphoinositides has been demonstrated in various tissues [14, 15], including endocrine pancreas [8, 16, 17]. Our results show that under conditions where carbachol increases glucose-induced insulin release, polyphosphoinositide breakdown, as evidenced by inositol phosphates accumulation, might be involved in the stimulus-secretion coupling. Cholinergic receptor activation in isolated islets of Langerhans results in mobilization of intracellular calcium [18, 19]. Inositol 1,4,5-trisphosphate has been suggested to be the second messenger capable of inducing calcium mobilization from the endoplasmic reticulum [20, 21] and the initial stimulation of calcium mobilization induced by carbachol could be due to receptor-mediated breakdown of polyphosphoinositides [18]. It remains difficult to assess the importance of this intracellular calcium mobilization in the control of insulin secretion; it seems however that it cannot by itself elicit this secretion [17, 22].

In contrast, the stimulation of insulin secretion induced by α,β-methylene ADP, a P_2 purinoreceptor agonist, was not accompanied by inositol phosphates accumulation in the islets. Among the different coupling mechanisms triggered off by the P_2 purinoreceptors, hydrolysis of membrane polyphosphoinositides has been demonstrated in hepatocytes [23, 24], Ehrlich ascites tumor cells [25] and aortic myocytes [26]. In these systems, the purinergic stimulation was associated with a rapid intracellular calcium mobilization, resulting in an increase in cytosolic calcium [24–27]. In the endocrine pancreas Morgan *et al.* [28] show that P_2 purinoreceptor activation with ATP, as well as stimulation of muscarinic receptors with carbachol, caused an increase in unidirectional $^{45}Ca^{2+}$ efflux from pre-loaded islets, interpreted to reflect mobilization of calcium from intracellular stores. Likewise, Hellman [5, 19] shows that exposure of islets to ATP results in a pronounced initial stimulation of $^{45}Ca^{2+}$ efflux, as noted when activating polyphosphoinositide breakdown with carbachol, provided that the Ca^{2+}-deficient perifusion medium is supplemented with glucose. Yet, our results are not in accordance with the involvement of polyphosphoinositide breakdown and seem to rule out this mechanism in the functional coupling of the P_2 purinoreceptors in the B cell. However, it must be noted that our experimental conditions markedly differ from those set by Hellman [5, 19].

A potentiating synergism between ADP or ATP and acetylcholine has been shown on insulin secretion from isolated perfused rat pancreas, in presence of a physiological glucose concentration [29]. In our experimental setting, in presence of a slightly stimulating glucose concentration, the effect of α,β-methylene ADP on insulin secretion was comparable to that of carbachol at the same concentration. The combined effect of both substances increased insulin secretion at a level compatible with a synergism of action, which, however, does not allow us to decide whether there is additivity or potentiation. However, the accumulation of inositol phosphates induced by both substances added simultaneously is not different from the accumulation induced by carbachol alone. Under our experimental conditions, purinergic stimulation by α,β-methylene ADP does not seem to interact with the effect of muscarinic stimulation on polyphosphoinositide breakdown.

In conclusion, both experimental series appear to rule out the involvement of polyphosphoinositide hydrolysis in the coupling mechanisms between activation of the P_2 purinoreceptors and insulin response of the B cell.

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