Anti-insulin antiserum increases inositol phosphate accumulation in rat pancreatic islets

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The role of insulin in modulating phosphoinositide breakdown and accumulation of inositol phosphates was investigated in isolated rat pancreatic islets by using GPAIS (guinea-pig anti-insulin antiserum) that neutralizes effects of insulin in the medium. At either 3.0 mM- or 16.7 mM-glucose or 3.0 mM-glucose plus 10 μ M-arecaidine propargyl ester (muscarinic receptor agonist), GPAIS (but not control serum) was able to increase InsP_2 and InsP_3 , but not InsP , in *myo*-[^3H] inositol-prelabelled islets. The effect of GPAIS on ^3H incorporation into InsP_3 was dose-dependent, with a half-maximal effect at a concentration able to bind 4004 ± 163 μ units of insulin. A specific mass assay of the biologically relevant isomer $\text{Ins}(1,4,5)\text{P}_3$ revealed a huge increase (> 3 -fold). Formation of PtdIns , PtdInsP and PtdInsP_2 was not affected by GPAIS. This is indirect evidence for an effect of insulin on inositide metabolism, and therefore endogenously released insulin may have led to an underestimation in earlier studies of effects of insulinotropic substances on inositol phosphate accumulation.

INTRODUCTION

It is widely recognized that hydrolysis of the membrane lipid PtdInsP_2 leads to the generation of the two intracellular messengers InsP_3 and diacylglycerol (Berridge & Irvine, 1984; Downes & Michell, 1985). The latter stimulates the phospholipid-dependent protein kinase C, whereas the InsP_3 produced, by mobilizing Ca^{2+} from intracellular stores, leads to activation of calmodulin and other Ca^{2+} -sensitive proteins, which has been extensively reviewed (Berridge, 1984; Farese, 1983). Glucose may also stimulate insulin secretion of rat pancreatic islets by activating these two branches of the Ca^{2+} -messenger system (Freinkel *et al.*, 1975; Laychock, 1983; Best *et al.*, 1987; Zawulich *et al.*, 1987). During a batch incubation of a large number of islets in a small volume, a high insulin concentration in the medium can be expected (Verspohl *et al.*, 1979); however, attention has not been paid to the possibility that this endogenously released insulin may have counter-regulatory effects on inositol turnover, thus obscuring glucose effects. This topic is interesting, since (first) insulin inhibits various glucose effects in islets and (second) insulin exhibits a negative feedback on insulin secretion (Verspohl *et al.*, 1979; Frerichs *et al.*, 1965), the mechanism of which is not completely understood.

By using GPAIS (guinea-pig anti-insulin antiserum), we aimed in this study to determine whether the released insulin might affect phosphoinositide breakdown and accumulation of inositol phosphates in isolated rat pancreatic islets. GPAIS has been proved an useful tool to increase the glucose effect on insulin release (Verspohl *et al.*, 1979) by neutralizing insulin in the medium, and by inhibiting, therefore, the well-known negative feedback of insulin on insulin secretion. By showing an increase in InsP_3 by GPAIS, the modulatory and hitherto not studied effect of insulin on inositol phosphates becomes obvious, and the data also provide indirect evidence for the mechanism of negative feedback of insulin.

EXPERIMENTAL

Animals

Wistar rats of either sex weighing 180–250 g were used. They

were kept on a standard pellet diet and tap water *ad libitum* at 22 °C with a 12 h-light/-dark cycle.

Chemicals

D-*myo*-[2- ^3H](n)Inositol (15.8 Ci/mmol) and a mixture of standards of [^3H]inositol phosphates were from New England Nuclear Corp. (Boston, MA, U.S.A.). The quantitative $\text{Ins}(1,4,5)\text{P}_3$ assay kit was from Amersham-Buchler (Braunschweig, Germany), collagenase (126–196 units/mg) from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), BSA (fatty acid free) from Behring Werke A.G. (Marburg, Germany), and Dowex-1 (100–200 mesh) from Serva, Heidelberg, Germany. Hepes, pilocarpine hydrochloride, soya-bean trypsin inhibitor, bacitracin and phosphoinositide standards were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). X-ray film X-Omat S was from Kodak A.G. (Stuttgart, Germany). Plastic t.l.c. sheets coated with silica gel 60, layer thickness 0.2 mm, were from Merck (Darmstadt, Germany). Arecaidine propargyl ester (APE) was given by Dr. E. Mutschler (Mutschler & Hultzs, 1973); this cholinergic agonist is selective for muscarinic receptors by not interacting with nicotinic receptors, and was shown to stimulate insulin release (Verspohl *et al.*, 1990).

Isolation of rat pancreatic islets

This was done as described by Lacy & Kostianovsky (1967), with slight modifications as described by Verspohl & Ammon (1980).

Measurement of PPI turnover

About 1000 islets which had been washed in Krebs–Ringer Hepes (KRH) buffer containing 0.1% BSA (fatty acid free) plus 3 mM-glucose were prelabelled for 2 h at 37 °C in 0.25 ml of KRH buffer containing 30 μ Ci of *myo*-[^3H]inositol in a conical centrifuge tube. After 110 min of incubation, when incorporation of [^3H]inositol into phospholipids approaches a steady state, LiCl was added to the mixture to give a final concentration of 10 mM. The islets were washed three times and centrifuged (100 g for 3 min) to remove radioactive medium. The washing medium was KRH buffer containing 10 mM-LiCl and 0.2 mM unlabelled

Abbreviations used: InsP_3 , *myo*-inositol trisphosphate (both isomers with positional determinants of the phosphate groups at either 1,4,5 or 1,3,4); GPAIS, guinea-pig anti-insulin antiserum; APE, arecaidine propargyl ester; KRH, Krebs–Ringer Hepes (118.4 mM-NaCl, 4.84 mM-KCl, 1.19 mM- KH_2PO_4 , 1.19 mM- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM-Hepes).

myo-inositol, but no radioactive material. Batches of 100 islets were then incubated in 0.4 ml of KRH buffer in the presence of unlabelled *myo*-inositol (1 mM) for 15 min to enable them to recover from the labelling and washing procedure. The islets were washed again once, and were thereafter incubated together with various concentrations of either test substance such as glucose or APE in the presence of either GPAIS or control serum (preimmunization serum) as indicated in the legends to give a final volume of 200 μ l. These experiments were performed in the presence of 10 mM-LiCl. The incubations were terminated after 180 s by adding 200 μ l of ice-cold 9% (w/v) HClO₄. The islets were sonicated (Econoclean; Spirig, Rapperswil, Switzerland) for 30 s (35 Hz, 150 W) and centrifuged (10000 *g* for 15 min) to precipitate proteins. The supernatant was extracted with 2 $\times$ 420 μ l of methanol/chloroform/12 M-HCl (200:100:1, by vol.). To the combined extraction fluids was added 400 μ l of chloroform and 200 μ l of water. Aqueous products containing inositol phosphates plus *myo*-inositol were separated from lipophilic products containing PtdIns and its phosphates by centrifugation (10000 *g* for 15 min); the lower organic phase containing the inositol phospholipids was dried under N₂.

Measurement of the water-soluble inositol phosphates released by hydrolysis of the phosphoinositides

The pH of the supernatants was adjusted to pH 8 by addition of a mixture of KOH (3 M) and sodium tetraborate (54 mM). Labelled InsP, InsP₂ and InsP₃ were separated from each other and from [³H]inositol by anion-exchange chromatography on 1 ml Dowex AG-X8 columns (formate form; 2 cm $\times$ 0.5 cm columns; 100–200 mesh) (Downes & Michell, 1981; Berridge *et al.*, 1983; Batty *et al.*, 1985). All fractions that were carefully monitored for their elution profile by using appropriate radioactively labelled internal standards were later combined, and portions were mixed with 15 ml of Aquasol Universal LSC Cocktail (NEN, Boston, MA, U.S.A.), and counted for radioactivity by liquid-scintillation spectrometry (Packard Isocap/300). Total labelled inositol phosphates were expressed as a percentage of total [³H]inositol lipids passed through the column.

Total amount of Ins(1,4,5)P₃

The absolute concentration of Ins(1,4,5)P₃, which is the

biologically relevant isomer, was measured by using a radioreceptor assay. In this, after a preincubation time 100 islets were incubated with the test compounds in 200 μ l for 180 s. The supernatant was then centrifuged at 12000 *g* for 15 min at 4 °C and the final supernatant was freeze-dried; after addition of 100 μ l of assay buffer, the samples were added to the Ins(1,4,5)P₃ assay mixture, containing a high specific radioactivity of labelled Ins(1,4,5)P₃ together with a specific Ins(1,4,5)P₃-binding protein.

Separation of inositol-containing phospholipids

The dried lower organic phase also containing precipitated proteins was reconstituted in 100 μ l of chloroform/methanol (9:1, v/v), and after 20 μ l had been used for the determination of total amount of phosphoinositides the remaining 80 μ l was applied to precoated silica-gel 60 t.l.c. plates. Standard solutions of PtdIns, PtdInsP and PtdInsP₂ were also applied, and the plate were developed as described by Schacht (1978). The plates were autoradiographed by using EN³HANCE (NEN), and afterwards radioactivity of cut plates corresponding to authentic phospholipids was counted.

Preparation of GPAIS

The procedure for this was that described by Verspohl *et al.* (1979); 1 μ l of GPAIS was able to bind 201 $\pm$ 8 μ units of insulin. For control experiments, preimmunization serum of guinea pigs was used. Because serum contains many different factors, i.e. various serum proteins, hormones (including insulin), proteases etc., precipitation with (NH₄)₂SO₄ was performed before freeze-drying. When GPAIS was added in experiments, an equivalent amount of fatty-acid-free BSA was omitted from the main incubation medium (total concentration of BSA and/or GPAIS was 0.5%).

Statistics

For statistical evaluation, multiple comparisons of means were carried out by two-way analysis of variance (F-ratio test) and subsequent Student's *t* test.

RESULTS

In Table 1 the effects of GPAIS, guinea-pig control serum and

Table 1. Effects of GPAIS, control serum (pre-immunization serum) and BSA buffer on [³H]inositol incorporation into phosphoinositides of rat pancreatic islets in the presence of various stimulants (3.0 mM and 16.7 mM-glucose; 3.0 mM-glucose plus 10 μ M-APE)

About 100 islets were incubated in medium containing [³H]inositol as described in the text. Main incubations in the presence of additions (GPAIS, control serum, BSA buffer) were performed for 180 s. When non-insulinotropic concentrations (3.0 mM) of glucose were present, 32 μ l of GPAIS or 32 μ l of control serum was added; in the other cases 96 μ l of sera was used. Labelled *myo*-inositol, InsP, InsP₂, and InsP₃ (aqueous fraction) add up to 100% of total radioactivity passed through the Dowex column. PtdIns, PtdInsP and PtdInsP₂ (lipophilic fraction) add up to 100% of total radioactivity on t.l.c. Values are the means $\pm$ S.E.M. for four independent experiments; * *P* < 0.01 versus control serum.

Stimulant	Addition	Incorporation (% of total)						
		Aqueous fraction				Lipophilic fraction		
		Ins	InsP	InsP ₂	InsP ₃	PtdIns	PtdInsP	PtdInsP ₂
3.0 mM-Glucose	GPAIS	87.1 $\pm$ 7.31	11.1 $\pm$ 1.41	1.39 $\pm$ 0.02*	0.54 $\pm$ 0.06*	99.9 $\pm$ 0.014	0.039 $\pm$ 0.002	0.017 $\pm$ 0.002
	Control serum	87.6 $\pm$ 3.51	10.8 $\pm$ 1.23	1.21 $\pm$ 0.03	0.27 $\pm$ 0.04	99.9 $\pm$ 0.009	0.040 $\pm$ 0.001	0.018 $\pm$ 0.001
	BSA buffer	87.4 $\pm$ 4.46	11.4 $\pm$ 1.83	0.88 $\pm$ 0.12	0.29 $\pm$ 0.06	99.9 $\pm$ 0.011	0.042 $\pm$ 0.004	0.018 $\pm$ 0.002
	GPAIS	86.0 $\pm$ 3.26	11.8 $\pm$ 1.97	1.28 $\pm$ 0.06*	0.79 $\pm$ 0.11*			
16.7 mM-Glucose	Control serum	87.4 $\pm$ 1.23	11.3 $\pm$ 0.93	1.01 $\pm$ 0.05	0.23 $\pm$ 0.06			
	BSA buffer	87.9 $\pm$ 4.18	10.9 $\pm$ 1.41	0.83 $\pm$ 0.06	0.42 $\pm$ 0.03			
	GPAIS	83.4 $\pm$ 6.21	13.3 $\pm$ 2.13	1.78 $\pm$ 0.02*	1.31 $\pm$ 0.08*			
3.0 mM-Glucose plus 10 μ M-APE	Control serum	85.6 $\pm$ 2.47	12.4 $\pm$ 2.86	1.38 $\pm$ 0.07	0.44 $\pm$ 0.09			
	BSA buffer	86.6 $\pm$ 1.19	11.7 $\pm$ 1.93	1.24 $\pm$ 0.09	0.53 $\pm$ 0.08			

Table 2. Effects of GPAIS, control serum (pre-immunization serum) and BSA buffer on $\text{Ins}(1,4,5)\text{P}_3$ content of rat pancreatic islets

Islets were incubated for 180 s in the presence of various glucose and APE concentrations. When non-insulinotropic concentrations (3.0 mM-glucose or 0.1 μM -APE) were present, 32 μl of GPAIS or 32 μl of control serum was added; in the other cases 96 μl of sera was used. $\text{Ins}(1,4,5)\text{P}_3$ was quantified as described in the Experimental section. Values are means $\pm$ S.E.M. for four experiments run in duplicate. N.S., not significant.

Addition	$\text{Ins}(1,4,5)\text{P}_3$ (pmol/100 islets)	<i>P</i> versus control serum
BSA buffer	0.109 ± 0.0445	N.S.
3.0 mM-Glucose		
GPAIS	0.414 ± 0.0497	< 0.001
Control serum	0.127 ± 0.0340	
8.3 mM-Glucose		
GPAIS	0.694 ± 0.0473	< 0.001
Control serum	0.189 ± 0.0276	
16.7 mM-Glucose		
GPAIS	1.131 ± 0.0942	< 0.001
Control serum	0.224 ± 0.0301	
0.1 μM -APE plus 3.0 mM-glucose		
GPAIS	0.402 ± 0.0296	< 0.001
Control serum	0.091 ± 0.0019	
10.0 μM -APE plus 3.0 mM-glucose		
GPAIS	1.331 ± 0.0713	< 0.001
Control serum	0.376 ± 0.0301	

BSA buffer on [^3H]inositol incorporation into phosphoinositides of rat islets is shown. The experiments were performed in the presence of either 3.0 mM- or 16.7 mM-glucose or 3.0 mM-glucose plus 10 μM -APE. In absolute terms, basal radioactivity (c.p.m.) (in presence of control serum) was 66 ± 9 (InsP_3), 327 ± 8 (InsP_2) and 587 ± 11 (InsP) per 100 islets. Both InsP_2 and InsP_3 were significantly increased by addition of 32 μl of GPAIS, independently of the stimulator used.

This effect of GPAIS on [^3H]inositol incorporation into InsP_3 was dose-dependent; radioactivity incorporation into the InsP_3 fraction was increased from 0.26 to 0.25, 0.31, 0.43 and 0.46% of total aqueous fraction by 3.2, 10, 32 and 100 μl of GPAIS respectively. The half-maximal effect was at about 20 μl of GPAIS, which was able to bind 4004 ± 163 μunits of insulin; this is about the amount of insulin leaked and secreted under basal conditions by 100 islets in our experiments.

There were no significant differences between the effects of GPAIS, control serum and BSA on PtdIns , PtdInsP and PtdInsP_2 at 3.0 mM-glucose (Table 1). Because of the small changes of PtdInsP_2 formation in general, it is hard to say whether polyphosphoinositide hydrolysis is the source of additional radioactivity incorporated into InsP_3 , which is the breakdown product of PtdInsP_2 .

In Table 2 the data on specific measurements of $\text{Ins}(1,4,5)\text{P}_3$ are summarized when GPAIS, guinea-pig control serum or BSA buffer was used. The data show that GPAIS significantly increases $\text{Ins}(1,4,5)\text{P}_3$ in islets compared with control serum, independently of the stimulator (glucose or APE) used. The maximal insulinotropic concentrations are 16.7 mM for glucose and 10 μM for APE (Verspohl *et al.*, 1990).

DISCUSSION

The data clearly show that GPAIS is able to enhance InsP_3 and InsP_2 accumulation, possibly owing to a decrease in

polyphosphoinositides in rat islets. Since by Dowex separation of inositol phosphates it is not possible to discriminate between the biological active $\text{Ins}(1,4,5)\text{P}_3$ and the biologically inactive $\text{Ins}(1,3,4)\text{P}_3$ isomer, the absolute amount of $\text{Ins}(1,4,5)\text{P}_3$ was also determined in islets. This newly introduced method eliminates ambiguous results owing to contamination by $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4 . The effect of GPAIS is probably due to its insulin-antagonizing effects, since it was observed independently of the stimulator, i.e. various glucose and APE concentrations used. Taken together, this is indirect evidence for a modulatory role of insulin on turnover of polyphosphoinositides: mainly the islet $\text{Ins}(1,4,5)\text{P}_3$ levels are under inhibitory influence of insulin.

The measured effects of glucose and other insulinotropic substances are therefore the resultant of an InsP_3 accumulation as described by others (Best & Malaisse, 1984; Best *et al.*, 1987; Zawulich *et al.*, 1987) and the inhibition of InsP_3 accumulation by simultaneously released insulin, as shown in the present study. This fits very well with the observation that a substance such as carbachol is more potent than glucose in increasing InsP_3 accumulation (Best *et al.*, 1987), since it is less insulinotropic than glucose (Hermans *et al.*, 1986). This may be interpreted by the fact that the modulatory effect of insulin in the medium on turnover of polyphosphoinositides is assumed to be less prominent. Interestingly, Zawulich *et al.* (1987) have shown that cholecystokinin increases InsP_3 , but mostly at low glucose concentrations, when again insulin in the medium is low. It is reasonable to assume that the hitherto measured and published effects of highly insulinotropic substances such as glucose on InsP_3 accumulation are small (1.5–4 times above basal) (Best & Malaisse, 1984; Best *et al.*, 1987; Zawulich *et al.*, 1987) and are more or less underestimated, owing to the presence of released insulin. In contrast, in other target tissues initiators are much more effective (e.g. carbachol 7-fold in rat parotid glands; (Berridge *et al.*, 1983).

Our study also contributes to the possible mechanism of negative feedback of insulin on insulin secretion (Ammon & Verspohl, 1976; Verspohl *et al.*, 1979), since other possibilities, such as decrease in cyclic AMP and inhibition of $^{45}\text{Ca}^{2+}$ uptake, all have proved negative (E. J. Verspohl, unpublished work).

In conclusion, the data obtained by using GPAIS show (first) that insulin increases phosphoinositide turnover, which is most obvious for InsP_3 accumulation, (second) that this effect is independent of the stimulator used (glucose or APE), and (third) that InsP_3 accumulation may have been underestimated in former investigations when high insulin concentrations owing to stimulators are present in the incubation medium. The observed decrease in inositol phosphate accumulation may be involved in the yet-undefined mechanism of various insulin effects on islets, e.g. inhibition of insulin release.

Financial support (Ve 90/2-2) by the Deutsche Forschungsgemeinschaft, Bonn, Germany, is greatly acknowledged.

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Received 27 September 1989/20 November 1989; accepted 30 November 1989