Possible involvement of a tyrosine kinase-dependent pathway in the regulation of phosphoinositide metabolism by vanadate in normal mouse islets

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The potential roles of protein tyrosine kinases (TKs) and of phosphotyrosine phosphatases (PTPs) in pancreatic islet function are not known. In this study, we investigated whether vanadate, a potent PTP inhibitor, affects phosphoinositide (PI) metabolism by a TK-dependent pathway in isolated mouse islets. To avoid the confounding effects of changes in Ca^{2+} influx, all experiments were performed in the absence of Ca^{2+} . In the presence of 15 mM glucose, vanadate, acetylcholine (ACh) or [Arg]vasopressin (AVP) strongly stimulated *InsP* production. Vanadate also increased *PtdInsP* levels in membranes. The TK inhibitor genistein (not its inactive analogues genistin and daidzein) significantly reduced vanadate effects, but was without effect in the absence of stimulation or in the presence of ACh or AVP. Islet proteins resolved by SDS/PAGE were analysed by immunoblotting with anti-phosphotyrosine antibody. Under control conditions, several phosphotyrosyl-proteins (PYPs) were

present. Vanadate increased phosphotyrosine residues on several PYPs, notably two proteins of 145 and 85 kDa. This effect was prevented by genistein. p145 and p85 could correspond to phospholipase $\text{C}\gamma$ (PLC γ) and the regulatory subunit of *PtdIns*-3-kinase (*PtdIns*-3K) respectively. Both proteins are expressed in islets, as revealed by immunoblots with specific antibodies. Tungstate, another PTP inhibitor, reproduced vanadate effects, but inhibition of *PtdIns*-3K by wortmannin failed to affect vanadate-increased *PtdInsP* levels. Incubation of the islets in the presence of 10 % (v/v) fetal calf serum instead of BSA increased *InsP* production and this effect was prevented by genistein. These results suggest that inhibition of PTP increases *InsP* production in mouse islets by a TK-dependent pathway. They also provide evidence for a potential role of TK and PTP in pancreatic B-cell function.

INTRODUCTION

The potential roles of protein tyrosine kinases (TKs) in pancreatic B-cells are not known. The presence of receptors for insulin-like growth factor 1 (IGF-1) and nerve-growth factor in islet cells [1,2], the sensitivity of islet cell replication to several growth factors [3], the identification of several TKs in islets [4] and the recent description of a receptor-type phosphotyrosine phosphatase (PTP) [5] closely related to a diabetes-specific islet autoantigen [6] suggest that TK and PTP could play important roles in the regulation of B-cell function. It has also recently been shown that genistein, a potent TK inhibitor [7], potentiates glucose-induced insulin release and inhibits prolactin-induced B-cell replication [8–10]. However, in contrast to its effect on B-cell replication [9], the acute effect of genistein on stimulus–secretion coupling is unlikely to result from an inhibition of TK [10].

The importance of phosphoinositide (PI) metabolism in the modulation of insulin secretion is well recognized. B-cells possess neurohormonal receptors coupled to G-proteins that can activate phospholipase C (PLC). This enzyme catalyses the hydrolysis of *PtdIns*(4,5) P_2 into *Ins*(1,4,5) P_3 and diacylglycerol. *Ins*(1,4,5) P_3 promotes mobilization of Ca^{2+} from the endoplasmic reticulum, while diacylglycerol activates protein kinase C [11]. Activation of this pathway underlies the potentiation of glucose-induced insulin release by acetylcholine (ACh), [Arg]vasopressin (AVP) or cholecystokinin [12–14]. Several isoforms of PLC have been described. While the activity of the β isoform (PLC β) is regulated by G-

protein-coupled receptors, the γ isoform (PLC γ) can be activated by several growth factors (for review, see [15]). However, it is not known whether PLC γ is expressed and plays a role in pancreatic islets.

Activation of TK by growth factors initiates a cascade of events culminating in specific cellular responses, such as increased cellular growth and replication [16]. Among others, two enzymes involved in the regulation of PI metabolism [PLC γ and *PtdIns*-3 kinase (*PtdIns*-3K)] can be activated by receptor-linked TK and generate intracellular signals involved in the specific response to growth factors [17]. *PtdIns*-3K catalyses the phosphorylation of *PtdIns* in the 3 position of the inositol ring, leading to the formation of *PtdIns*3 P , *PtdIns*(3,4) P_2 and *PtdIns*(3,4,5) P_3 , whose precise roles as second messengers have still to be determined [18]. Both enzymes are probably also activated by non-receptor TK [19].

Vanadate, a PTP inhibitor [20], increases PI metabolism in mouse pancreatic islets [21]. In other tissues, pervanadate increases PI metabolism and protein tyrosine phosphorylation concomitantly [22], and both effects are ascribed to the ability of pervanadate to inhibit PTP [23,24]. In the present study, we have thus compared the effects of vanadate, ACh and AVP to determine whether islet PI metabolism can be regulated not only by G-protein-coupled receptors, but also by a TK-dependent pathway. We then determined whether pancreatic islets are equipped with PLC γ and *PtdIns*-3K, two proteins involved in this pathway. Since vanadate and TK inhibitors may affect Ca^{2+}

Abbreviations used: TK, protein tyrosine kinase; PTP, phosphotyrosine phosphatase; PYP, phosphotyrosyl-protein; PI, phosphoinositide; ACh, acetylcholine; AVP, [Arg]vasopressin; PLC, phospholipase C; *PtdIns*-3K, *PtdIns*-3-kinase; FCS, fetal calf serum; PVDF, poly(vinylidene difluoride); ECL, enhanced chemiluminescence; NP-40, Nonidet P-40; IGF-1, insulin-like growth factor 1.

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influx in pancreatic B-cells [10,21], all experiments were performed in the absence of extracellular Ca^{2+} to avoid the confounding effect of changes in Ca^{2+} influx on PI metabolism.

MATERIALS AND METHODS

Materials

Genistein was obtained from Calbiochem-Behring (San Diego, CA, U.S.A.), daidzein from either Carl Roth GmbH (Karlsruhe, Germany) or LC Laboratories (Woburn, MA, U.S.A.) and heat-inactivated fetal calf serum (FCS) from Gibco Brl Life Technologies GmbH (Eggenstein, Germany). Genistin, wortmannin, sodium tungstate, acetylcholine chloride, Nonidet P-40 (NP-40) and protease inhibitors were from Sigma (St. Louis, MO, U.S.A.), AVP was from Peninsula Laboratories (Belmont, CA, U.S.A.) and sodium orthovanadate (Na_3VO_4) from Janssen Chimica (Beerse, Belgium). Stock solutions of genistein, genistin, daidzein (50–100 mM) and wortmannin (2 mM) were prepared in DMSO. Control islets were exposed to the same concentration of DMSO as treated islets ($< 0.2\%$). Gel electrophoresis reagents of the highest purity grade were obtained from Bio-Rad (Munich, Germany), poly(vinylidene difluoride) (PVDF) membranes were from Dupont NEN (Boston, MA, U.S.A.) and mouse monoclonal anti-phosphotyrosine antibody PY20 was obtained from Leinco Technologies Inc. (St. Louis, MO, U.S.A.). Mouse monoclonal antibody against PLC γ and rabbit polyclonal antibody against the regulatory subunit of PtdIns-3K (p85 PtdIns-3K) were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Secondary peroxidase-conjugated antibodies, enhanced chemiluminescence (ECL) detection reagents and *myo*-[2- ^3H]inositol were purchased from Amersham (Amersham, Bucks., U.K.).

Preparation

All experiments were carried out at 37 °C with islets obtained by collagenase digestion of the pancreas of fed female NMRI mice.

Solutions

Except when otherwise stated, the medium used was a bicarbonate-buffered solution containing: 120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 and 24 mM NaHCO_3 . It was gassed with O_2/CO_2 (94/6, v/v) to maintain pH 7.4 and was supplemented with 1 mg/ml BSA fraction V (Boehringer, Mannheim, Germany). The final incubations of the islets were carried out in a nominally Ca^{2+} -free medium prepared by replacing CaCl_2 with MgCl_2 .

Measurements of PI metabolism

After isolation, the islets were loaded with *myo*-[2- ^3H]inositol for 2 h in the presence of 15 mM glucose. After washing in the presence of 1 mM unlabelled inositol, batches of 40–50 islets were incubated for 30 min in 0.5 ml of medium without CaCl_2 containing 10 mM LiCl, 1 mM unlabelled inositol and 15 mM glucose, and supplemented or not with a TK inhibitor. Twofold concentrated test substances were then added in 0.5 ml of prewarmed medium, and the islets were incubated for a further 30 min period. At the end of the incubation, the experiment was stopped by addition of 3 ml of CHCl_3 /methanol/concentrated HCl (200/100/1, by vol.) and 100 μl of a 100 mM EDTA solution. The water and lipid phases were then separated by centrifugation. Free inositol and inositol phosphates dissolved in the water phase were separated by anion-exchange

chromatography as described previously [25,26]. PIs labelled with *myo*-[2- ^3H]inositol (lipid phase) were measured after deacylation and separation of the glycerophosphoesters as described [14,27]. Because these experiments were performed in non-equilibrium conditions, no conclusion could be drawn about the absolute levels of InsP or PI.

Measurements of PYPs in islets

After isolation, the islets were preincubated for 60 min in the presence of 15 mM glucose. They were then incubated, in batches of 80–100, in 0.5 ml of medium without CaCl_2 containing 15 mM glucose and supplemented or not with a TK inhibitor. At appropriate times, 0.5 ml of prewarmed medium supplemented with twofold concentrated test substances was added to the tubes, and the islets were further incubated for 1, 5, 15 or 30 min, so that the total incubation always lasted 60 min. At the end of the incubation, the islets were sedimented by rapid centrifugation (5 s) and the medium was removed. The islets were then rinsed once with 1 ml of ice-cold PBS containing 200 μM vanadate and 100 μM EDTA. After a rapid vortex and centrifugation (5 s), PBS was removed and the islets were resuspended in 100 μl of lysis buffer supplemented with 200 μM vanadate. Islet lysates were then prepared and SDS/PAGE and immunoblotting were performed as described in the following sections.

Preparation of islet homogenates and lysates

For islet homogenate preparation, the islets were suspended (100/100 μl) in ice-cold lysis buffer of the following composition: 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EGTa, 1 mM EDTA, 1% NP-40, leupeptin and aprotinin (2 $\mu\text{g}/\text{ml}$ of each), and 100 $\mu\text{g}/\text{ml}$ of freshly added PMSF. They were then sonicated and stored at -70°C . For islet lysate preparation, the islets were suspended in ice-cold lysis buffer, incubated for 30 min at 4 °C, briefly sonicated and cleared by centrifugation for 10 min at 10000 g. The supernatant (lysate) was removed and kept at -70°C . In some experiments, the pellet was resuspended in lysis buffer by sonication and kept at -70°C . The protein concentration was determined in each sample by the method of Bradford using BSA as standard [28].

Gel electrophoresis and immunoblotting

Samples were mixed with concentrated (3 $\times$) Laemmli sample buffer [29] and boiled for 5 min. Approx. 150 μg of lysate proteins were then electrophoresed on a 6.5 or 7.5% polyacrylamide gel under reducing conditions. Electrophoretic transfer of proteins to PVDF membranes was performed using the technique of Bjerrum and Schafer-Nielsen [30], in the presence of 370 mg/l SDS. After staining of proteins with Ponceau S, the membrane was destained and incubated for 2 h in Tris-buffered saline (TBS: 50 mM Tris, pH 7.4, 200 mM NaCl and 0.05% Tween 20) supplemented with 30 mg/ml BSA. The membrane was then incubated with anti-PLC γ antibody (1 $\mu\text{g}/\text{ml}$ TBS + BSA), anti-(p85 PtdIns-3K) antibody (0.25 $\mu\text{g}/\text{ml}$ TBS + BSA), or anti-phosphotyrosine antibody (0.2–0.4 $\mu\text{g}/\text{ml}$ TBS + BSA), either overnight at 4 °C or for 1 h at room temperature. After five washes with TBS, detection of bound antibodies was carried out with the ECL kit from Amersham using Kodak X-AR films. For some experiments, antibodies were removed from membranes by heating at 55 °C for 30 min in sodium phosphate buffer (5 mM, pH 7.5) containing 20 mg/ml SDS and 2 mM 2-mercaptoethanol [31]. After extensive washes with TBS, the membrane could be reprobed with another antibody. Band intensities were determined by densitometry analysis of autoradiograms.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. for the indicated number of experiments (different animals or islet preparations) or batches of islets. The statistical significance of differences between means was assessed by analysis of variance followed by a test of Newman–Keuls. Differences were considered significant at $P < 0.05$.

RESULTS

Comparison of the effects of vanadate, tungstate, ACh and AVP on PI metabolism

Islets preincubated with *myo*-[2-³H]inositol to label their PIs were incubated with various agents in a Ca²⁺-free medium containing 15 mM glucose and 10 mM LiCl (to inhibit InsP phosphatases). As shown in Table 1(A), 1 mM vanadate increased InsP and InsP₂ levels, and tended to increase InsP₃ levels, as already described [21]. Vanadate also doubled PtdInsP without affecting PtdIns and PtdInsP₂ levels (Table 1A). Similar

effects of vanadate were observed in the presence of a non-stimulatory (3 mM) concentration of glucose (results not shown).

In another set of experiments, we compared the effects of vanadate with those of tungstate, another PTP inhibitor [32]. As shown in Table 1(B), qualitatively similar effects were observed with both inhibitors, although tungstate was less potent than vanadate. However, a significant increase in PtdInsP₂ levels was produced by tungstate, but not vanadate.

ACh (100 μ M) and AVP (1 μ M), two agents that stimulate PLC activity through their binding to a specific G-protein-coupled receptor, also increased InsP and InsP₂ levels under these conditions (Table 1C) [14]. However, ACh and AVP did not significantly affect the levels of PI in islets, but there was a trend to a decrease in PtdIns levels.

Effects of TK inhibition on the stimulation of PI metabolism by vanadate

If vanadate increases PI metabolism by inhibiting PTP, its effect should be attenuated by pretreatment with a TK inhibitor such as genistein [33]. As shown in Table 2(B), genistein pretreatment

Table 1 Comparison of vanadate, tungstate, ACh and AVP effects on phosphoinositide metabolism in incubated mouse islets

After preincubation with *myo*-[2-³H]inositol, the islets were washed and subdivided in batches of 40–50. Each batch was then rinsed with a medium without CaCl₂ before being transferred into 0.5 ml of medium containing 15 mM glucose, no CaCl₂, 1 mM inositol and 10 mM LiCl. After 30 min incubation at 37 °C, 0.5 ml of prewarmed medium was added to each tube. This medium was identical to that in which islets were already incubated (controls), or supplemented with twofold concentrated test substances. Thirty min later, the incubation was stopped, water and lipid phases were separated, and labelled inositol phosphates and phosphoinositides were extracted. Inositol phosphates were separated by anion-exchange chromatography and labelled phosphoinositides were measured after deacylation and separation of the glycerophosphoesters (see the Materials and methods section). Values are means $\pm$ S.E.M. for the indicated number of batches of islets.

Experimental conditions		<i>n</i>	InsP (d.p.m./islet)	InsP ₂ (d.p.m./islet)	InsP ₃ (d.p.m./islet)	<i>n</i>	PtdIns (d.p.m./islet)	PtdInsP (d.p.m./islet)	PtdInsP ₂ (d.p.m./islet)
A	Controls	21	25 $\pm$ 1	10 $\pm$ 1	10 $\pm$ 1	21	1565 $\pm$ 82	33 $\pm$ 2	21 $\pm$ 1
	Vanadate (1 mM)	22	97 $\pm$ 4 ^b	41 $\pm$ 2 ^b	13 $\pm$ 1 ^a	22	1534 $\pm$ 79	74 $\pm$ 4 ^b	23 $\pm$ 1
B	Controls	6	25 $\pm$ 2	11 $\pm$ 1	11 $\pm$ 1	6	1598 $\pm$ 81	40 $\pm$ 2	21 $\pm$ 1
	Vanadate (1 mM)	6	100 $\pm$ 7 ^b	41 $\pm$ 4 ^b	12 $\pm$ 1	6	1517 $\pm$ 124	80 $\pm$ 7 ^b	21 $\pm$ 2
	Tungstate (5 mM)	6	99 $\pm$ 9 ^b	21 $\pm$ 2 ^c	8 $\pm$ 1	6	1672 $\pm$ 139	60 $\pm$ 4 ^a	27 $\pm$ 2 ^c
C	Controls	10	31 $\pm$ 2	11 $\pm$ 1	10 $\pm$ 1	3	1468 $\pm$ 134	28 $\pm$ 2	21 $\pm$ 1
	ACh (100 μ M)	10	558 $\pm$ 46 ^b	34 $\pm$ 3 ^b	12 $\pm$ 1	3	1277 $\pm$ 64	29 $\pm$ 1	21 $\pm$ 2
	AVP (1 μ M)	8	173 $\pm$ 12 ^b	24 $\pm$ 2 ^b	10 $\pm$ 1	4	1327 $\pm$ 177	28 $\pm$ 4	20 $\pm$ 2

^a $P < 0.01$; ^b $P < 0.001$; ^c $P < 0.05$ versus controls by ANOVA + Newman–Keuls.

Table 2 Effects of TK inhibition on control and vanadate-stimulated phosphoinositide metabolism in incubated mouse islets

After preincubation with *myo*-[2-³H]inositol, the islets were washed and subdivided in batches of 40–50. Each batch was then rinsed with a medium without CaCl₂ before being transferred into 0.5 ml of medium containing 15 mM glucose, no CaCl₂, 1 mM inositol and 10 mM LiCl, and supplemented as indicated with 100 μ M genistein or its inactive analogues daidzein and genistin. After 30 min incubation at 37 °C, 0.5 ml of prewarmed medium was added to each tube. This medium was (A) identical to that in which islets were already incubated, or (B) supplemented with concentrated 2 mM vanadate (to achieve a final concentration of 1 mM). Thirty min later, the incubation was stopped, water and lipid phases were separated and processed as described in Table 1. Values are means $\pm$ S.E.M. for the indicated number of batches of islets.

Experimental conditions	<i>n</i>	InsP (d.p.m./islet)	InsP ₂ (d.p.m./islet)	InsP ₃ (d.p.m./islet)	PtdIns (d.p.m./islet)	PtdInsP (d.p.m./islet)	PtdInsP ₂ (d.p.m./islet)
(A) No vanadate							
—	7	26 $\pm$ 3	10 $\pm$ 1	10 $\pm$ 1	1530 $\pm$ 147	29 $\pm$ 3	21 $\pm$ 1
Genistein	7	21 $\pm$ 1	10 $\pm$ 1	9 $\pm$ 1	1583 $\pm$ 160	31 $\pm$ 4	21 $\pm$ 2
Daidzein	7	19 $\pm$ 1	9 $\pm$ 1	9 $\pm$ 1	1527 $\pm$ 117	28 $\pm$ 2	22 $\pm$ 1
Genistin	4	24 $\pm$ 3	10 $\pm$ 2	10 $\pm$ 2	1521 $\pm$ 246	31 $\pm$ 4	24 $\pm$ 3
(B) Vanadate (1 mM)							
—	7	95 $\pm$ 9	40 $\pm$ 4	13 $\pm$ 1	1503 $\pm$ 85	65 $\pm$ 5	24 $\pm$ 2
Genistein	7	49 $\pm$ 6 ^b	18 $\pm$ 2 ^b	9 $\pm$ 1	1572 $\pm$ 114	50 $\pm$ 4 ^c	23 $\pm$ 2
Daidzein	7	70 $\pm$ 8 ^a	35 $\pm$ 4	12 $\pm$ 1	1525 $\pm$ 131	57 $\pm$ 5	22 $\pm$ 1
Genistin	5	97 $\pm$ 9	41 $\pm$ 4	13 $\pm$ 1	1480 $\pm$ 349	64 $\pm$ 4	24 $\pm$ 2

^a $P < 0.01$; ^b $P < 0.001$; ^c $P < 0.05$ versus controls by ANOVA + Newman–Keuls.

Figure 1 Effects of vanadate on PYPs in pancreatic islets

After preincubation, islets were subdivided in batches of 80–100. Each batch was then rinsed with a medium containing 15 mM glucose without CaCl_2 and transferred into 0.5 ml of the same medium. After incubation for 59, 55 or 45 min at 37 °C, 0.5 ml of prewarmed medium was added to the tubes. This medium was identical to that in which islets were already incubated (controls, lane 1), or supplemented with 2 mM vanadate (to achieve a final concentration of 1 mM) (lanes 2–4). The incubation was prolonged for 1, 5 or 15 min as indicated, to reach a total incubation time of 1 h. The reaction was stopped and islet lysates were prepared as described in the Materials and methods section. Islet proteins ($\sim 150 \mu\text{g}$) were then resolved by 6.5% SDS/PAGE, transferred to PVDF membranes and immunoblotted with anti-phosphotyrosine antibody. The position of molecular-mass markers is indicated on the right, and major vanadate-induced PYPs on the left. The results are representative of at least three experiments.

Figure 2 Scanning densitometer analysis of PYPs from islets incubated in the presence of vanadate

In (A) traces were obtained by scanning densitometer analysis of immunoblots illustrated in Figure 1 (6.5% SDS/PAGE). Vanadate treatment for 15 min (dotted line) increased phosphotyrosine residues in several proteins compared with control islets (solid line). Part of the trace corresponding to the upper part of the blot (≥ 190 kDa) has been omitted. In (B) the traces correspond to immunoblots shown in Figure 3 (7.5% SDS/PAGE). In the presence of genistein (dotted line), vanadate-induced increase in phosphotyrosine residues was less marked for several proteins compared with vanadate-treated islets in the absence of genistein (continuous line). p60, p85, p106 and p145 show the position of PYPs of 60, 85, 106 and 145 kDa. Note that the difference in protein migration between traces in (A) and (B) is due to a difference in polyacrylamide gel concentration.

Figure 3 Effects of the TK inhibitor genistein on vanadate-induced PYPs in pancreatic islets

The experiments were performed as described in Figure 1, except for the following items. When present, genistein (100 μM) was added to the medium throughout the 1 h incubation. Vanadate was added as indicated, after 45 min of incubation, for a further 15 min incubation. Islet proteins were resolved by 7.5% SDS/PAGE. The position of molecular-mass markers is indicated on the right, and major vanadate-induced PYPs on the left. The results are representative of four experiments out of five.

reduced vanadate effects on InsP and InsP_2 levels by two-thirds, and those on PtdInsP levels by 40%. The effects of genistein were not reproduced by its analogues which are inactive on TK, i.e. genistin or daidzein [34], although a slight inhibition of InsP levels was observed with daidzein. The different test substances were ineffective when PI metabolism was not stimulated (Table 2A) or was stimulated by ACh or AVP (results not shown).

These results suggest that the effects of vanadate on PI metabolism, but not those of ACh or AVP, are mediated by a TK-dependent pathway. In other cell types, it has been demonstrated that activation of TK can stimulate two enzymes involved in PI metabolism, $\text{PLC}\gamma$ and PtdIns-3K [17]. The following experiments were aimed at investigating whether this pathway is also present in islets and could underlie the activation of islet PI metabolism produced by vanadate.

Effects of vanadate on PYPs in pancreatic islets

Inhibition of PTP by vanadate may be expected to increase phosphotyrosine residues in proteins of islet cells, as in other cell types [23]. We studied PYPs in pancreatic islets by SDS/PAGE followed by immunoblotting with a mouse monoclonal anti-phosphotyrosine antibody. Under control conditions (15 mM glucose without CaCl_2), several PYPs were observed, including a major protein of 106 kDa (Figures 1–3). The general pattern of PYPs under control conditions was reproducible with only slight variations. For instance, in some experiments, a protein of ~ 120 kDa was much more tyrosine-phosphorylated, so that p117, p122 and p125 (Figure 1) could not be clearly distinguished from each other (see Figures 3 and 5). Vanadate induced a clear increase in phosphotyrosine residues in several PYPs, listed on

Figure 4 Detection of PLC γ and p85 PtdIns-3K in pancreatic islets

Proteins were resolved by 7.5% SDS/PAGE (minigel) and transferred to PVDF membranes that were subsequently cut in two parts at the level of migration of the 116 kDa molecular-mass marker. The upper part was then immunoblotted with a mouse monoclonal anti-PLC γ antibody and the lower part with a rabbit polyclonal anti-(p85 PtdIns-3K) antibody. The origin and amount of blotted proteins were: lane 1, A431 cell homogenate (10 μ g); lane 2, islet homogenate (20 μ g); lanes 3–5, islet lysate (10, 20 and 40 μ g); lane 6, pellet formed during clarification of islet lysate (15 μ g). The position of molecular-mass markers is indicated on the right. The results are representative of two individual experiments.

the left of Figure 1 (lanes 2–4). The major changes were observed for proteins of 60, 74, 79, 85, 110, 117, 125, 145 and 165 kDa. Several PYPs were not affected by vanadate treatment, notably p106 and a protein of 90–94 kDa (Figures 1, 2A and 3). A clear increase in the phosphorylation state of a protein of 185 kDa could also be observed when ECL detection was prolonged, but not in all experiments. The effect of vanadate was roughly the same after 1, 5 or 15 min treatment (Figure 1) and persisted after 30 min treatment (results not shown).

The reproducibility of vanadate effects on PYPs was not 100%: in three out of 17 experiments, there was no increase in phosphotyrosyl residues after 15 min treatment with vanadate, despite the presence of a normal pattern of PYPs. Out of the 14 experiments in which vanadate increased phosphotyrosyl residues in islet proteins, there was no increase in the phosphorylation state of p145 in three experiments in which p145 was detected. In three other experiments, proteins higher than 130 kDa were not detectable, probably because of the low amount of proteins resolved on SDS/PAGE or a problem of transfer in these particular experiments.

Figure 3 shows that genistein, which did not affect PYPs under control conditions, partially prevented vanadate effects on several PYPs. This effect, observed in four out of five experiments, is also illustrated in Figure 2(B). It clearly shows that genistein reduced the effects of vanadate on p74, p79, p85 and p145 while p106, that was not tyrosine-phosphorylated in response to vanadate (Figure 2A), remained unaffected by the TK inhibitor.

Like vanadate, 5 mM tungstate (15 min) increased phosphotyrosine residues in several islet proteins and particularly in proteins of 85 and 145 kDa, whereas ACh and AVP (15 min) did not consistently affect PYPs in islets (results not shown).

Identification of PLC γ and PtdIns-3K in mouse pancreatic islets

Of particular interest were two PYPs of 145 and 85 kDa whose phosphorylation state was increased by vanadate and tungstate. Their molecular mass corresponds to that of PLC γ and the regulatory subunit of PtdIns-3K (p85 PtdIns-3K), respectively. The presence of both proteins in islets was confirmed by immunoblots with specific antibodies (Figure 4), and both

Figure 5 Co-migration of p85 PtdIns-3K and vanadate-induced p85 PYPs in pancreatic islets

Islets were incubated under control conditions (C) or treated with 1 mM vanadate for 15 min (V) as described in the legend to Figure 1, before being lysed. Proteins (40 μ g) were then resolved by 7.5% SDS/PAGE (minigel), transferred to PVDF membrane, and PYPs were revealed by immunoblotting with anti-phosphotyrosine antibody (right panel). Bound antibodies were then removed at 55 °C in the presence of 2-mercaptoethanol. After extensive washes, the membrane was reblotted with anti-(p85 PtdIns-3K) antibody (left-hand panel). The position of molecular-mass markers is indicated on the right. The arrows indicate the position of p85 on both blots.

Figure 6 Effects of PtdIns-3K inhibition on vanadate-stimulated phosphoinositide metabolism in incubated mouse islets

After preincubation with *myo*-[2-³H]inositol, the islets were washed and subdivided into batches of 40–50. Each batch was then rinsed with a medium without CaCl₂ before being transferred into 0.5 ml of medium containing 15 mM glucose, no CaCl₂, 1 mM inositol and 10 mM LiCl. The medium was also supplemented with the indicated concentration of wortmannin. After 30 min of incubation at 37 °C, 0.5 ml of prewarmed medium was added to each tube. This medium was identical to that in which islets were already incubated (controls), or supplemented with 2 mM vanadate (to achieve a final concentration of 1 mM). Thirty min later, the incubation was stopped, water and lipid phases were separated and processed as described in Table 1. Results are expressed as a percentage of control values without vanadate: 20 ± 2 , 10 ± 2 , 10 ± 2 and 40 ± 9 d.p.m./islet for InsP, InsP₂, InsP₃ and PtdInsP respectively. Wortmannin did not affect PtdIns or PtdInsP₂ levels. Results are means $\pm$ S.E.M. for five separate experiments.

proteins were completely extracted in islet lysates, as shown by their absence in the proteins of the pellet obtained by clarification of islet homogenates (Figure 4, lane 6).

To determine whether vanadate-induced PYP p85 could correspond to the regulatory subunit of PtdIns-3K, successive immunoblots were performed with anti-phosphotyrosine and anti-(p85 PtdIns-3K) antibodies. These showed that PYP p85 and p85 PtdIns-3K exactly co-migrated on the gel (Figure 5). Unfortunately, attempts to immunoprecipitate PLC γ and p85 PtdIns-3K from islet lysates were unsuccessful, due to the small amount of tissue available.

Effects of PtdIns-3K inhibition with wortmannin on PI metabolism

We then tested the effects of wortmannin, a potent inhibitor of PtdIns-3K [35], on PI metabolism. As shown in Figure 6, wortmannin prevented vanadate-induced increase in PtdIns P levels only at 1 μ M. This high concentration of wortmannin also prevented the stimulation of Ins P production by vanadate (Figure 6) or by ACh or AVP (results not shown).

Effects of FCS on islet PI metabolism

As a first approach to evaluating the physiological relevance of our findings, we investigated whether factors present in FCS influence islet PI metabolism through a TK-linked pathway. For the reasons explained above, the experiments were carried out in the absence of extracellular Ca^{2+} . A 30 min treatment of the islets with 10% (v/v) FCS (with 250 μ M EGTA to chelate the Ca^{2+} contained in FCS) instead of BSA (7 mg/ml to mimic the high protein concentration carried by FCS) increased the level of Ins P by 50% (36.3 ± 3.3 versus 24.6 ± 1.8 d.p.m./islet; $P < 0.01$ by Student's t -test, $n = 8$). In the presence of 100 μ M genistein, FCS was without effect on Ins P levels (28.0 ± 3.0 d.p.m./islet versus 24.7 ± 2.6 d.p.m./islet in controls; $n = 5$).

DISCUSSION

This study shows that pancreatic islets possess two enzymes involved in the regulation of PI metabolism by TK, PLC γ and PtdIns-3K. The latter enzyme is a heterodimer composed of a regulatory (p85) and a catalytic (p110) subunit. Although we did not directly evaluate the presence of the catalytic subunit in islets, expression of the regulatory subunit p85 PtdIns-3K and of PLC γ in mouse pancreatic islets was revealed by immunoblotting with specific antibodies. Furthermore, we provide some functional evidence that both proteins can be activated by a TK-dependent pathway in islets.

ACh, AVP and vanadate are known to increase Ins P production in islets, both in the absence and presence of extracellular Ca^{2+} [12,14,21]. However, their effects are larger in the presence of extracellular Ca^{2+} , probably because islet PLC activity is Ca^{2+} -dependent [36]. In this study, therefore, Ca^{2+} was omitted from the medium to avoid PI metabolism being affected secondary to changes in Ca^{2+} influx produced by vanadate. It is important to note that vanadate does not increase cytoplasmic Ca^{2+} concentration in the absence of extracellular Ca^{2+} [21].

Several observations indicate that vanadate increases islet PI metabolism by mechanisms different from those of ACh and AVP. First, the effect of vanadate was significantly reduced by genistein, a potent inhibitor of TK, but not by its inactive analogues genistin and daidzein. These results contrast with the lack of effect of genistein on Ins P production induced by AVP and ACh, which activate PLC β through binding to their specific G-protein-coupled receptor [37,38]. Secondly, unlike ACh and AVP, vanadate also increased the level of PtdIns P and this effect was significantly reduced by genistein, but not by genistin or daidzein. The possibility that the effects of vanadate are related

to its ability to inhibit PTP in islets was further substantiated by the observation that tungstate, another PTP inhibitor, produced qualitatively similar effects on both Ins P production and PtdIns P levels. Although the method used did not allow us to distinguish between different isomers of PtdIns P and PtdIns P_2 , we suggest that the increase in PtdIns P (and PtdIns P_2) levels produced by PTP inhibitors could correspond to an increase in PtdIns3 P [and PtdIns(3,4) P_2], produced by PtdIns-3K, for the following reasons. First, these products are not substrates for PLC [18] and they could thus accumulate in islets even in the presence of an increased PLC activity, while PtdIns4 P and PtdIns(4,5) P_2 are expected to decrease slightly [12,14]. Secondly, products of PtdIns-3K have recently been detected by HPLC in rat pancreatic islets and an insulin-secreting B-cell line [39]. Thirdly, the regulatory subunit of PtdIns-3K has been clearly identified in islets. All these results lend support to the hypothesis that PTP inhibitors can activate PLC γ and PtdIns-3K by a TK-dependent pathway in pancreatic islets.

Measurements of PYPs in islets somehow reinforce this hypothesis. Vanadate increased phosphotyrosine residues in several islet proteins, and this effect was reduced by genistein. These proteins have not been identified in this study, but we note that they have a similar molecular mass to some PYPs observed in response to vanadate or pervanadate in other cell types [24,40,41]. p60 could correspond to pp60 c-src, p90–94 to the β subunit of the insulin- or IGF-1 receptor, p125 to the focal-adhesion kinase and p165 to the platelet-derived growth factor or epidermal growth factor receptor monomer, while p185 could correspond to the insulin-receptor-substrate 1. Of particular interest for the present study was the increase in phosphotyrosine residues in p85 and p145, that could correspond to the regulatory subunit of PtdIns-3K and to PLC γ . Unfortunately, attempts to immunoprecipitate these proteins from mouse pancreatic islets were unsuccessful, due to the limited amount of tissue available.

Phosphorylation of PLC γ on tyrosine residues increases its activity and translocates the enzyme from the cytosol to the membrane [15]. An increase in phosphotyrosine residues on PLC γ was observed in response to vanadate in several cell types, and this effect occurred concomitantly with an increase in Ins P production [22,23,42]. Although we have no direct proof that p145 PYP is effectively PLC γ , our results strongly suggest that a similar mechanism of PLC γ activation exists in mouse pancreatic islets. This hypothesis is reinforced by the recent observation that the vascular endothelial growth factor increases phosphotyrosine content of PLC γ in the tumoral insulin-secreting cell line RINm5F [4].

Activation of PtdIns-3K by vanadate has been shown in permeabilized platelets, where a production of PtdIns(3,4) P_2 was detected concomitantly with a decrease in PtdIns(4,5) P_2 levels due to PLC activation [24]. In our study, vanadate increased phosphotyrosine residues in a protein of 85 kDa that exactly co-migrated with p85 PtdIns-3K on SDS/PAGE, suggesting that p85 PYP is p85 PtdIns-3K. However, it is unclear whether the increase in PtdIns-3K activity brought about by various TKs necessarily requires tyrosine-phosphorylation of the p85 regulatory subunit [18]. Vanadate also increased PtdIns P levels and both effects of vanadate were reduced by an inhibitor of TK. On the other hand, wortmannin, a potent inhibitor of PtdIns-3K ($\text{EC}_{50} \sim 5$ nM) [35], did not specifically inhibit the increase in PtdIns P levels produced by vanadate in pancreatic islets. It is also theoretically possible that vanadate increased PtdIns P levels through inhibition of a PtdIns P phosphodiesterase, as in the pituitary GH $_4$ C $_1$ cell line [43]. Taken together, our results are compatible with, but do not prove, activation of PtdIns-3K by a TK-dependent pathway in pancreatic islets.

The effects of micromolar concentrations of wortmannin on PI metabolism cannot be attributed to PtdIns-3K inhibition, because they also occurred in the absence of stimulatory agents and during stimulation with ACh or AVP. These high concentrations of wortmannin have been reported to inhibit agonist-stimulated InsP_3 formation by inhibiting the myosin-light-chain kinase in adrenal glomerulosa cells [44]. An inhibition of InsP production could explain the inhibition by micromolar amounts of wortmannin of insulin release in the insulin-secreting HIT cells stimulated by nutrients and bombesin [45].

In other cell types, certain neuropeptides that stimulate PLC activity by a G-protein-dependent mechanism (e.g. AVP and bombesin) have been shown to increase phosphotyrosine residues in several proteins [46,47]. In our study, a 15 min treatment with ACh or AVP did not increase phosphotyrosine residues in pancreatic islets, in contrast to vanadate. However, we cannot exclude the possibility that their effect on islet PYPs was very transient.

The physiological implication of our observation has still to be determined. Pancreatic islets respond to a wide variety of growth factors (see the Introduction and [3]). In view of our results, it is tempting to speculate that $\text{PLC}\gamma$ and PtdIns-3K could play an important role in mediating effects of the growth factors in islet cells. The effects of FCS on islet PI metabolism and their inhibition by genistein suggest that some as yet unidentified factor(s) present in serum can influence PI metabolism via TK. Activation of $\text{PLC}\gamma$, like that of $\text{PLC}\beta$, also leads to the generation of $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol. As these second messengers potentiate glucose-induced insulin release in pancreatic islets, TK could also modulate insulin secretion by regulating $\text{PLC}\gamma$ activity. The products of PtdIns-3K have recently been shown to be potent activators of an atypical isoform of protein kinase C, zeta [48]. Interestingly, this isoform of protein kinase C is expressed in pancreatic islets and in several insulin-secreting cell lines [49]. Further investigation of this pathway could provide interesting information on the control of islet cell proliferation and differentiation, and on the regulation of insulin release.

This work was supported by Grant 3.42525.94 from the Fonds de la Recherche Scientifique Médicale, Brussels and by Grant SPPS-AC 89/95-135 from the Ministry of Scientific Policy, Brussels. J.-C. J. is 'Chargé de Recherches' of the Fonds National de la Recherche Scientifique, Brussels.

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