Tyrosine kinases play a permissive role in glucose-induced insulin secretion from adult rat islets

S J Persaud, T E Harris, C J Burns and P M Jones

Cellular and Molecular Endocrinology Group, Biomedical Sciences Division, King's College London, Campden Hill Road, London W8 7AH, UK

(Requests for offprints should be addressed to S J Persaud)

ABSTRACT

The role(s) played by protein tyrosine kinases (PTKs) in the regulation of insulin secretion from pancreatic β cells is not clear. We have examined the effects of glucose, the major physiological insulin secretagogue, on the tyrosine phosphorylation state of islet proteins, and assessed β cell insulin secretory responses in the presence of PTK inhibitors. Under basal conditions islets contained many proteins phosphorylated on tyrosine residues, and glucose (20 mM; 5–15 min) was without demonstrable effect on the pattern of tyrosine phosphorylation, in either the absence or presence of the protein tyrosine phosphatase (PTP) inhibitor, sodium pervanadate (PV). PV alone (100 μ M) increased tyrosine phosphorylation of several islet proteins. The PTK inhibitors genistein (GS) and tyrphostin A47

(TA47) inhibited islet tyrosine kinase activities and glucose-, 4 α ketoisocaproic acid (KIC)- and sulphonylurea-stimulated insulin release, without affecting glucose metabolism. GS and TA47 also inhibited protein serine/threonine kinase activities to a limited extent, but had no effect on Ca^{2+} , cyclic AMP- or phorbol myristate acetate (PMA)-induced insulin secretion from electrically permeabilised islets. These results suggest that PTK inhibitors exert their inhibitory effects on insulin secretion proximal to Ca^{2+} entry and it is proposed that they act at the site of the voltage-dependent Ca^{2+} channel which regulates Ca^{2+} influx into β cells following nutrient- and sulphonylurea-induced depolarisation.

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INTRODUCTION

The intracellular mechanisms regulating insulin secretion from pancreatic β cells are an active area of endocrine research and the roles played by serine/threonine protein kinases in this process have been studied extensively (reviewed by Persaud *et al.* 1994). However, much less is known about the involvement of protein tyrosine kinases (PTKs) in β cell signal transduction processes, although a picture of PTKs present in islets and β cells and some idea of the identities of their substrate proteins is beginning to emerge. Thus it is clear that β cells of normal islets and β cell lines express a number of receptor and cytoplasmic PTKs (Öberg *et al.* 1994, Kanaka-Gantenbein *et al.* 1995, Öberg-Welsh & Welsh 1995, Sorenson & Stout 1995, Harbeck *et al.* 1996, Sekine *et al.* 1996) and several proteins which are known to play signal transduction roles in other cell types are phosphorylated on tyrosine residues in islets or β cells (Rothenberg *et al.* 1995, Jonas & Henquin 1996, Persaud *et al.* 1996, Sekine

et al. 1996). There is convincing evidence that PTKs are important for β cell differentiation from endocrine precursor cells, with reports of identification of particular PTKs in β cells and growth of foetal pancreatic cells following their activation (Öberg *et al.* 1994, Kanaka-Gantenbein *et al.* 1995, Otonkoski *et al.* 1996).

However, there is not yet a clear consensus as to whether activation of PTKs modulates the insulin secretory response, nor to the intracellular sites of regulation by tyrosine phosphorylation. Some of the confusion may arise from the multiple pharmacological actions, unrelated to tyrosine kinase inhibition, of commonly used PTK inhibitors (Young *et al.* 1993). In a study using normal islets from adult rodents, genistein (GS) stimulated insulin secretion, but its inactive analogue, daidzein, produced similar effects, indicating a dissociation between PTK inhibition and stimulation of insulin secretion (Jonas *et al.* 1995). In the same study, tyrphostin A47 (TA47) significantly inhibited glucose-stimulated insulin secretion. Further

confusion may arise from comparing responses of insulin-secreting cell lines and foetal or neonatal islets with those of adult islets containing fully differentiated β cells, since the protein expression and secretory responses of proliferating β cells may not always reflect the situation in normal adult islets. For example, a comparison of PTKs amplified from cDNAs from the RINm5F β cell line, foetal islets and adult islets indicated that different PTKs were expressed in the different preparations (Öberg *et al.* 1994). In addition, studies using neonatal islets (Sorenson *et al.* 1994), MIN6 (Ohno *et al.* 1993) or insulin secreting cell line (INS-1) (Verspohl *et al.* 1995) β cell lines have indicated that inhibition of PTKs with membrane permeant inhibitors enhances insulin release, perhaps suggestive of a tonic inhibitory role for PTKs on secretion from proliferating β cells. In contrast, there has been one report, using the β TC3 cell line, that PTK inhibition causes inhibition of both tyrosine phosphorylation and insulin secretion in response to glucose and the cholinergic agonist carbachol (Konrad *et al.* 1996). The situation is further complicated by reports, using cell lines and neonatal and adult islets, that receptor-mediated activation of PTKs either stimulates (Brelje *et al.* 1994) or has no effect on (Sekine *et al.* 1996, Tazi *et al.* 1996) insulin release, while inhibition of protein tyrosine phosphatases (PTPs) has no effect on basal secretion and augments glucose-stimulated insulin release (Zhang *et al.* 1991, Persaud *et al.* 1996). These observations are inconsistent with a tonic inhibitory role for PTKs in insulin secretion. In light of this general confusion on the signalling roles of PTKs in the regulation of insulin secretion, we have now examined tyrosine phosphorylation and insulin secretory responses of normal adult islets, to determine the involvement of islet PTKs in the physiological secretory response of fully differentiated β cells in adult rat islets of Langerhans.

MATERIALS AND METHODS

Materials

Collagenase (type XI), BSA (fraction V), hydrogen peroxide, dimethylthiazol-diphenyl tetrazolium bromide (MTT), glibenclamide, tolbutamide, phenyl methyl sulphonyl fluoride (PMSF), leupeptin, histone (type IIA and IIIS), myosin light chains, 4 β phorbol myristate acetate (PMA), cyclic AMP and isobutyl methylxanthine (IBMX) were purchased from Sigma Chemical Co. (Poole, Dorset, UK). GS, daidzein and TA47 and TA1 were obtained from Calbiochem (Beeston, Nottingham, UK).

Monoclonal anti-phosphotyrosine IgG was from Upstate Biotechnology Inc. (NY, USA) and goat anti-mouse IgG was obtained from Dako Ltd (High Wycombe, Bucks, UK). Enhanced chemiluminescence (ECL) reagents, Rainbow protein molecular weight markers and [125 I] for insulin iodination were from Amersham International (Amersham, Bucks, UK) and [γ 32 P]ATP (3000 Ci/mmol) was from DuPont (UK) Ltd (Stevenage, Herts, UK). All other reagents were of analytical grade from BDH (Poole, Dorset, UK). Rats (Sprague–Dawley; 250g) were supplied by King's College London Animal Unit. The MIN6 β cell line was kindly provided by Professor J I Miyazaki and Dr Y Oka (University of Tokyo, Japan).

Insulin secretion

Islets of Langerhans were isolated from rat pancreata by collagenase digestion as previously described (Jones *et al.* 1993), and for some experiments they were electrically permeabilised, in a buffer mimicking the intracellular environment, by exposure to a high intensity electric field (Jones *et al.* 1985). In static incubation secretion experiments, groups of three intact or five permeabilised islets were incubated for 1 h with secretagogues in the absence or presence of PTK inhibitors. The time-course of insulin secretory responses and the reversibility of the effects of TA47 were determined in a temperature-controlled (37 °C) perfusion system: groups of 40 intact islets were picked into perfusion chambers, and equilibrated for 30 min at a flow rate of 1 ml/min in a non-stimulatory physiological salt solution (Gey & Gey 1936), after which fractions were collected every 2 min for analysis of insulin content. Immunoreactive insulin was measured in all experiments by radioimmunoassay as previously described (Jones *et al.* 1988).

Immunological detection of tyrosine phosphorylated islet proteins

Groups of 100–300 islets or 1.5×10^6 MIN6 β cells were incubated for 5–15 min at 37 °C in the presence of 2 or 20 mM glucose in the absence or presence of sodium pervanadate (PV) or PTK inhibitors. Islets or β cells were pelleted, disrupted by sonication in gel electrophoresis sample buffer (Jones *et al.* 1988), and proteins were resolved on 10% polyacrylamide gels. Fractionated proteins were electrophoretically transferred onto 0.2 μ m nitrocellulose membranes which were blocked overnight in phosphate buffered saline (PBS) supplemented with 0.05% (v/v) Tween 20 and 1% (w/v) BSA and then incubated overnight in

the presence of 1 µg/ml mouse monoclonal anti-phosphotyrosine antibody. Membranes were washed (4x15 min) in PBS/0.05% (v/v) Tween-20 then incubated with peroxidase-conjugated goat anti-mouse IgGs (250 ng/ml) for 2 h. Tyrosine-phosphorylated proteins were detected by ECL following binding of the goat anti-mouse IgGs to the anti-phosphotyrosine antibody and their molecular masses were determined from a calibration curve constructed from the migration positions of coloured protein molecular mass markers.

Islet glucose metabolism

The effects of PTK inhibitors on glucose metabolism were determined by the MTT colorimetric assay in which the rate of MTT reduction to formazan crystals correlates with glucose oxidation and glucose utilisation (Jancic & Wollheim 1992). Groups of 10 islets were picked into wells of microtitre plates, preincubated for 1 h in the absence of MTT, then for a further hour with 1 mg/ml MTT at 2 or 20 mM glucose in the absence or presence of GS or TA47. Formazan crystals were solubilised in isopropanol and the optical density was recorded with an automated plate reader at 570 nm. Changes in background absorbance, in the absence of islets, were corrected for by subtraction from the appropriate optical density values obtained in the presence of islets.

Measurement of effects of PTK inhibitors on islet serine/threonine kinase activities

For measurement of protein kinase C (PKC), protein kinase A (PKA) and Ca^{2+} /calmodulin-dependent protein kinase (CaMK) activities, islets were resuspended in a lysis buffer (20 mM Tris/HCl (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, 50 µg/ml leupeptin, 1 mM PMSF and 0.1% (v/v) 2-mercaptoethanol) and disrupted by sonication (MSE Soniprobe, 3x15 s, 6 µ). PKA and CaMK activities of sonicated islet extracts were measured in the presence of appropriate activators and substrates (100 µM cyclic AMP, 100 µM IBMX, 1 mg/ml histone IIA and 50 µg/ml calmodulin, 0.5 mM CaCl_2 , 1 mg/ml myosin light chains respectively) and 100 µM [$\gamma^{32}\text{P}$]ATP as previously described (Persaud & Jones 1995). For measurement of islet PKC activity, PKC was partially purified from sonicated islet extracts by anion exchange chromatography and ^{32}P incorporation into histone H1S (1 mg/ml) was determined in the presence of 96 µg/ml phosphatidylserine, 6.4 µg/ml diolein and 1.3 mM CaCl_2 (Persaud & Jones 1995).

FIGURE 1. Immunological detection of tyrosine-phosphorylated islet proteins. Groups of 300 (left lane) or 100 (right lane) islets were incubated for 5 min in the absence (left lane) or presence (right lane) of 100 µM of the PTP inhibitor, PV. Islet proteins were separated on 10% gels and tyrosine phosphorylated proteins were detected by immunoblotting. Numbers indicate migration positions of molecular mass markers (kDa). Results are representative of three separate experiments.

Statistical analysis

Results are presented, where appropriate, as means $\pm$ S.E.M. for the number of observations as indicated. Statistical significance was determined by one way analysis of variance, followed by Bonferroni's multiple comparisons test where necessary. All other data were analysed by Student's *t*-tests or by Welch's *t*-tests for data means with different standard deviations. Differences between experimental and control samples were considered significant at $P < 0.05$.

RESULTS

Tyrosine phosphorylation of islet proteins

Under non-stimulatory conditions (2 mM glucose), islets expressed several tyrosine kinase substrates, the most notable of which had approximate molecular masses of 33, 34, 42, 44, 48, 51, 55, 90 and 112 kDa (Fig. 1, left lane). Exposure of islets

to the PTP inhibitor, PV (100 μ M), resulted in an overall increase in tyrosine phosphorylation, together with the appearance of some novel tyrosine-phosphorylated substrates (Fig. 1, right lane; increased phosphorylation of proteins of approximate molecular masses of 36, 37, 39, 42, 51, 55, 59, 72–85, 115–125 and 145 kDa). Although PV caused marked increases in the phosphotyrosine content of unstimulated islets, its effects on insulin secretion were minimal: in only one of three experiments did it significantly stimulate secretion (2 mM glucose: 0.23 ± 0.05 ng/islet/h; 2 mM glucose+100 μ M PV: 0.58 ± 0.07 , $P < 0.01$, $n=7$), and the secretory response was considerably less than that stimulated by 20 mM glucose in the same experiment (7.30 ± 0.63 ng/islet/h). When islets were exposed to 20 mM glucose for 15 min, the profile of tyrosine phosphorylated proteins was not detectably different from that seen in the presence of 2 mM glucose (Fig. 2A). In similar experiments, carried out in the presence of 100 μ M PV to inhibit PTPs, 20 mM glucose was without marked effect on the tyrosine phosphorylation state of islet proteins when determined after 5, 10 and 15 min exposure (Fig. 2B).

Effects of tyrosine kinase inhibitors on insulin secretion

The lack of effect of glucose on tyrosine phosphorylation, together with the dissociation between the large effects of PV on phosphotyrosine content of islet proteins and its small effects on insulin secretion, make it difficult to draw conclusions about the requirement for signalling through PTK pathways for insulin secretion based on measurements of tyrosine phosphorylation alone. The effects of pharmacological modulation of PTK activities on islet and β cell secretory responses to insulin secretagogues were therefore examined. Table 1 shows that two structurally dissimilar

FIGURE 2. Effect of glucose on islet PTK activities. The figure shows tyrosine phosphorylated islet proteins and the numbers indicate the migration positions of molecular mass markers (kDa). Results are representative of four separate experiments. (A) Groups of 300 islets were incubated for 15 min in the presence of 2 mM (left lane) or 20 mM (right lane) glucose. (B) Groups of 100 islets (lanes 1–4) or 50 islets (lanes 5 and 6) were incubated for 5 (lanes 1 and 2), 10 (lanes 3 and 4) or 15 (lanes 5 and 6) min in the presence of 2 mM glucose (lanes 1, 3 and 5) or 20 mM (lanes 2, 4 and 6) glucose. All incubations were carried out in the presence of 100 μ M PV.

TABLE 1. Effects of PTK inhibitors on glucose-stimulated insulin secretion. Intact islets were incubated for 1 h at 2 mM glucose or at 20 mM glucose $\pm$ PTK inhibitors or their inactive analogues and insulin secretion was measured by radioimmunoassay. Data are taken from four independent experiments, each of 8–9 observations and secretion is expressed as a percentage of stimulated (20 mM) insulin secretion. From the four experiments, mean insulin secretion at 2 mM glucose was 0.10 ± 0.009 ng/islet/h and at 20 mM glucose it was 2.82 ± 0.32 ng/islet/h

Treatment	Insulin secretion (% glucose-stimulated)
50 μ M genistein	136 ± 7^a
100 μ M genistein	96 ± 12
200 μ M genistein	34 ± 4^b
500 μ M genistein	3 ± 0.3^b
500 μ M daidzein	100 ± 7
12.5 μ M TA47	80 ± 6^a
25 μ M TA47	74 ± 3^a
50 μ M TA47	40 ± 4^b
100 μ M TA47	48 ± 6^b
200 μ M TA47	5 ± 1^b
12.5 μ M TA1	98 ± 8
25 μ M TA1	141 ± 17^a
50 μ M TA1	143 ± 10^a

^a $P < 0.05$, ^b $P < 0.0001$ versus secretion at 20 mM glucose.

inhibitors of PTKs, GS and TA47, both caused dose-related inhibition of glucose-stimulated insulin secretion, such that at the highest concentrations used, secretion was not significantly different from that obtained at a substimulatory concentration of glucose (2 mM). GS (50 μ M) caused a small, but significant, potentiation of glucose-stimulated secretion, but at concentrations of GS greater than 100 μ M, secretion was significantly inhibited. The inactive analogue of GS, daidzein, was without effect on insulin release when used at a concentration (500 μ M) at which GS fully inhibited glucose-stimulated secretion. Similar effects were obtained with TA47, which was a more potent inhibitor of glucose-induced insulin release, causing maximal inhibition at 200 μ M. The inactive analogue of TA47, TA1, did not inhibit secretion, but low concentrations (25 and 50 μ M) significantly potentiated the response to glucose (Table 1). Pretreatment of islets with 100 μ M PV for 10 min, to inactivate PTPs, did not significantly affect the inhibition of secretion caused by GS and TA47 (20 mM glucose: 5.42 ng/islet/h; +500 μ M GS: 0.41 ± 0.08 ; +100 μ M PV+500 μ M GS: 0.55 ± 0.16 ; +200 μ M TA47: 0.17 ± 0.05 ; +100 μ M PV+200 μ M TA47: 0.30 ± 0.08 , $n=9$, all $P < 0.001$ versus 20 mM glucose). The maximal inhibitory concentrations of GS and TA47 also fully inhibited insulin

secretion in response to 4 α ketoisocaproic acid (KIC), a leucine metabolite (2 mM glucose: 0.23 ± 0.05 ng/islet/h; 10 mM KIC: 3.79 ± 0.35 ; 10 mM KIC+500 μ M GS: 0.31 ± 0.02 ; 10 mM KIC+200 μ M TA47: 0.45 ± 0.09 , $n=7-8$, $P < 0.001$ versus 10 mM KIC).

The inhibition of insulin secretion from islets did not result from irreversible damage to the β cells since the effects were fully reversible. It can be seen from the perfusion experiments shown in Fig. 3A that the inhibitory effect of TA47 was rapid in onset and full inhibition of the secretory response to 20 mM glucose was achieved within 20 min. In addition, the TA47-induced inhibition was rapidly reversible upon its removal (Fig. 3B). Thus, in the presence of 20 mM glucose and 200 μ M TA47, secretion was only marginally higher than basal (min 10–30 versus 0–10), but replacement of the medium with 20 mM glucose alone (min 30–50) resulted in the expected rapid and biphasic secretory response.

Since glucose-stimulated insulin secretion is dependent on metabolism of glucose, and PTK inhibitors have been shown to inhibit metabolic enzymes (Young *et al.* 1993), it is possible that the inhibitory effects of GS and TA47 on glucose-stimulated insulin release could reflect inhibition of glucose metabolism. However, using the MTT assay which has been established for assessing glucose metabolism in insulin-secreting cells (Jancic & Wollheim 1992), neither GS nor TA47 significantly affected glucose-stimulated formazan production from MTT (2 mM glucose: 0.068 ± 0.009 absorbance units; 20 mM glucose: 0.271 ± 0.026 ; +500 μ M GS: 0.298 ± 0.007 ; +200 μ M TA47: 0.255 ± 0.004 , $n=5$, $P > 0.2$ versus 20 mM glucose for GS and TA47). In addition, GS and TA47 also inhibited insulin secretion in response to the sulphonylureas tolbutamide and glibenclamide, which stimulate insulin release by inhibiting ATP-sensitive K^+ channels. A maximal inhibitory concentration of GS fully inhibited tolbutamide- and glibenclamide-stimulated secretion at a threshold stimulatory concentration of glucose (5 mM), while TA47 caused a significant, but incomplete, inhibition of secretion in response to the sulphonylureas (200 μ M tolbutamide: $628 \pm 100\%$ secretion at 5 mM glucose; +500 μ M GS: $86 \pm 14\%$; +200 μ M TA47: $271 \pm 51\%$; 10 μ M glibenclamide: $528 \pm 71\%$ secretion at 5 mM glucose; +500 μ M GS: $157 \pm 37\%$; +200 μ M TA47: $271 \pm 66\%$, $n=9$, $P < 0.05$ for both PTK inhibitors). Under the same experimental conditions GS inhibited 20 mM glucose-stimulated insulin secretion to basal levels while there was still a small residual response in the presence of TA47 (5 mM

FIGURE 3. Time-course and reversibility of the inhibition of glucose-stimulated insulin secretion by TA47. Islets were perfused with 2 mM glucose for 30 min before the start of the experiments and basal secretion measured over the last 10 min is shown in both panels A and B (at min 0–10). Arrows indicate times at which perfusion media were changed as shown. Data for each panel are mean secretion ($\pm$ S.E.M.) from four separate groups of islets. (A) At min 10–30: 20 mM glucose; min 30–50: 20 mM glucose+200 μ M TA47. (B) At min 10–30: 20 mM glucose+200 μ M TA47; min 30–50: 20 mM glucose.

glucose: 0.07 ± 0.01 ng/islet/h; 20 mM glucose: 2.57 ± 0.31 ; +500 μ M GS: 0.12 ± 0.02 ; +200 μ M TA47: 0.32 ± 0.02 .

FIGURE 4. Inhibition of PTK activities by GS and TA47. The figure shows tyrosine phosphorylated islet (panel A) and MIN6 β cell (panel B) proteins, detected as described in Materials and Methods. The numbers indicate molecular masses (kDa). Results are representative of four separate experiments. Treatments were the same for islets (200 islets/lane) and MIN6 cells (120 μ g protein/lane), with incubations being performed in the absence of PV (lane 1) or in the presence of 100 μ M PV (lanes 2–6). Lane 1: 2 mM glucose; lane 2: 2 mM glucose; lane 3: 2 mM glucose+200 μ M GS; lane 4: 2 mM glucose+500 μ M GS; lane 5: 2 mM glucose+50 μ M TA47; lane 6: 2 mM glucose+200 μ M TA47.

Effects of tyrosine kinase inhibitors on islet tyrosine kinase and serine/threonine kinase activities

The effects of TA47 and GS on tyrosine phosphorylation of endogenous islet proteins was examined by treating islets for 5 min with GS and TA47 at concentrations which partially (200 μ M GS, 50 μ M TA47) or maximally (500 μ M GS, 200 μ M TA47) inhibited insulin secretion. It can be seen from Fig. 4A that inclusion of 100 μ M PV led to marked increases in the tyrosine phosphorylation state of numerous proteins (lane 2 versus lane 1), and that exposure to either GS or TA47 for 5 min

caused a concentration-dependent decrease in the phosphotyrosine content of several of these proteins, with the effects of TA47 (lanes 5 and 6) being more marked than those of GS (lanes 3 and 4). The high level of tyrosine phosphorylation obtained in the presence of PV obscures, to a certain extent, the inhibitory effects of the PTK inhibitors on the phosphorylation of individual β cell proteins, but it can be seen clearly from Fig. 4B that a 90 kDa β cell PTK substrate was almost completely dephosphorylated in the presence of 500 μ M GS (lane 4) or 200 μ M TA47 (lane 6). Although GS and TA47 clearly inhibited β cell PTK activities, the concentrations required for this and for full inhibition of glucose-stimulated insulin secretion were relatively high and it is known that high concentrations of both compounds may inhibit serine/threonine kinases (O'Dell *et al.* 1991). To address this possibility, the effects of GS and TA47 on islet PKC, PKA and CaMK activities *in vitro* were assessed. Both GS and TA47 significantly inhibited islet PKC activity (control: 128 ± 3.8 fmol/islet/min; +500 μ M GS: 68 ± 4.2 ; +200 μ M TA47: 51 ± 1.2 , $n=4$), while TA47, but not GS, inhibited PKA activity (control: 70 ± 1.9 fmol/islet/min; +500 μ M GS: 74 ± 0.9 ; +200 μ M TA47: 32 ± 0.6 , $n=4$). In both instances, the inhibitory effects of GS and TA47 were partial. In the CaMK assay, Ca^{2+} and calmodulin stimulated the phosphorylation of both exogenous myosin light chains and endogenous islet proteins of molecular masses ranging from 33 to 60 kDa (Fig. 5). In the presence of 500 μ M GS, islet CaMK activity was not noticeably affected, but 200 μ M TA47 caused a marked decrease in phosphorylation of both exogenous and endogenous substrates. Again, although profound, the inhibitory effect of TA47 was not complete, as phosphorylation in its presence was still greater than that obtained in the absence of Ca^{2+} and calmodulin. In particular, phosphorylation of the 57–60 kDa substrates, which may reflect autophosphorylation of endogenous CaMK, was still greatly enhanced.

Effects of tyrosine kinase inhibitors on insulin secretion from electrically permeabilised islets

Insulin secretion from electrically permeabilised islets was stimulated by direct activation of PKC, PKA and CaMK by PMA, cyclic AMP and Ca^{2+} respectively (Table 2). The responses to these agents were not inhibited by GS or TA47, when used at concentrations which fully inhibited glucose-stimulated insulin secretion.

FIGURE 5. Effects of GS and TA47 on islet Ca^{2+} /calmodulin-dependent kinase activity. The figure shows an autoradiograph of ^{32}P incorporation into islet proteins in the absence (lane 1) or presence (lanes 2–4) of Ca^{2+} and calmodulin. Islet extracts were incubated for 10 min in the absence of TK inhibitors (lanes 1 and 2) or in the presence of 500 μ M GS (lane 3) or 200 μ M TA47 (lane 4). Numbers indicate the molecular masses (kDa) of some endogenous islet proteins which showed Ca^{2+} /calmodulin-dependent phosphorylation, and MLC indicates phosphorylation of exogenous myosin light chains.

DISCUSSION

In the present study, the importance of PTK-mediated signalling pathways in the rapid insulin secretory events of pancreatic β cells has been examined. It is clear that PTK/PTP signal transduction systems are active in unstimulated islets since, in the absence of exogenous stimuli, numerous tyrosine-phosphorylated proteins were identified and inhibition of PTPs with PV caused a marked increase in the tyrosine phosphorylation state of many islet proteins, indicative of a rapid

TABLE 2. Effects of TA47 and GS on insulin secretion from electrically permeabilised islets. Islets were permeabilised, incubated for 1 h as below and insulin secretion was determined by radioimmunoassay. The effect of cyclic AMP and PMA were determined in the presence of 50 nM Ca^{2+} . Insulin secretion is expressed as pg/islet/h ($\pm$ s.e.m.), and the numbers of observations are shown in parentheses

Treatment	Insulin secretion (pg/islet/h)
50 nM Ca^{2+}	176 $\pm$ 12 (18)
10 μM Ca^{2+}	490 $\pm$ 16 ^a (18)
10 μM Ca^{2+} +200 μM TA47	431 $\pm$ 35 ^{a,b} (9)
10 μM Ca^{2+} +500 μM GS	533 $\pm$ 32 ^{a,b} (9)
500 μM cyclic AMP	442 $\pm$ 31 ^a (18)
500 μM cyclic AMP+200 μM TA47	501 $\pm$ 36 ^{a,b} (9)
500 μM cyclic AMP+500 μM GS	428 $\pm$ 26 ^{a,b} (9)
500 nM PMA	517 $\pm$ 18 ^a (18)
500 nM PMA+200 μM TA47	478 $\pm$ 28 ^{a,b} (9)
500 nM PMA+500 μM GS	512 $\pm$ 34 ^{a,b} (9)

^a $P < 0.0001$ versus secretion at 50 nM Ca^{2+} , ^b $P > 0.1$ versus appropriate control in absence of PTK inhibitor.

phosphorylation/dephosphorylation cycle of tyrosine residues. It has been reported recently that exposure of mouse islets to vanadate for 15 min resulted in enhanced tyrosine phosphorylation of proteins of molecular masses 60, 74, 79, 85 110, 117 125, 145 and 165 kDa (Jonas & Henquin 1996) and these observations fit in well with the findings of the present study, in which proteins of similar molecular masses, and some of lower molecular masses, showed increased phosphorylation on tyrosine residues following inhibition of PTPs. However, in the current experiments there were no marked glucose-dependent changes in the tyrosine phosphorylation state of islet PTK substrates, in keeping with an earlier report that glucose-responsive tyrosine phosphorylation is absent in islets (Rothenberg *et al.* 1995). Although it is impossible to rule out glucose-induced changes in the phosphotyrosine content of minority substrates, the dissociation between the large increases in tyrosine phosphorylation in response to PV and its small effects on insulin secretion suggest that it is difficult to define a role for PTKs in the regulation of insulin release from measurements of changes in tyrosine phosphorylation alone.

A complementary approach, that of measurement of the insulin-secretory capacity of islets and β cells in the presence of PTK inhibitors, has been reported previously by several groups (Ohno *et al.* 1993, Sorenson *et al.* 1994, Jonas *et al.* 1995, Verspohl *et al.* 1995). In the present study, 50 μM GS significantly potentiated glucose-stimulated

insulin release from rat islets, as did low concentrations of the inactive analogue of TA47, TA1, but higher concentrations of GS and all concentrations of TA47 were inhibitory. Two earlier reports (Ohno *et al.* 1993, Jonas *et al.* 1995) have suggested that the stimulatory effects of GS on insulin secretion may occur independently of PTK inhibition, and it is possible that GS-induced increases in cyclic AMP (Ohno *et al.* 1993) may account for the potentiation of insulin secretion seen at low concentrations of this PTK inhibitor, but that this stimulatory effect is masked by PTK inhibition at higher concentrations of GS. Our data indicate that high concentrations of PTK inhibitors are required to inhibit tyrosine phosphorylation of endogenous proteins, and suggest that the true and consistent effect of inhibition of PTKs in adult rat islets is an inhibition of glucose-stimulated insulin secretion. The inhibitory effect of 100 μM TA47 in the present studies was identical to that reported previously for mouse islets (Jonas *et al.* 1995) and perfusion studies confirmed that TA47 causes a rapid and reversible inhibition of secretion in response to 20 mM glucose. Examination of the effects PTK inhibitors on serine/threonine kinases indicated that GS and TA47 were also able to inhibit islet PKC, PKA and CaMK activities to varying extents. TA47 was the more promiscuous inhibitor, causing marked, but incomplete, inhibition of all three kinases. GS inhibited islet PKC activity, without affecting activities of PKA and CaMK. The partial inhibitory effects of PTK inhibitors on serine/threonine kinase activities was insufficient to inhibit secretion in response to activators of these kinases, as the secretory responses of electrically permeabilised islets to Ca^{2+} , PMA and cyclic AMP were not affected by the presence of PTK inhibitors. Nor can the inhibition of glucose-stimulated insulin release be explained by the inhibitory effects of the PTK inhibitors on serine/threonine kinases, as the common inhibitory effect of GS and TA47 was on islet diacylglycerol-sensitive PKC activity, but this would not be expected to fully inhibit glucose-induced insulin release (reviewed by Persaud *et al.* 1992).

The inhibitory effects of the PTK inhibitors on insulin secretion could not be ascribed to inhibition of glucose metabolism, because they also inhibited secretion stimulated by KIC, which acts distal to glycolysis, and glucose-stimulated formazan production, an index of glucose oxidation and utilisation, was not affected by GS or TA47. Both inhibitors also significantly reduced tolbutamide- and glibenclamide-induced insulin release at a threshold stimulatory concentration of glucose. These results give an indication of the site of action

of PTK inhibitors on the insulin secretory process. Since they inhibited glucose-, KIC- and sulphonylurea-stimulated insulin release, but not Ca^{2+} -induced secretion, it is clear that GS and TA47 exert their effects distal to closure of ATP-sensitive K^+ channels, but proximal to increases in intracellular Ca^{2+} . A site of action at the voltage-dependent Ca^{2+} channel (VDCC) would be consistent with these data, and a previous report indicated that GS reversibly inhibited voltage-activated Ca^{2+} currents in β cells from adult mouse islets (Jonas *et al.* 1995). The mode of action of GS on blockade of β cell Ca^{2+} channels has not been established, but since we have found that two structurally dissimilar PTK inhibitors inhibited insulin secretion at a site proximal to increased Ca^{2+} levels, it is likely that they block the channels as a consequence of PTK inhibition rather than by a physical inhibition of Ca^{2+} influx. The likelihood of a non-physical interaction of the PTK inhibitors with Ca^{2+} channels is supported by the lack of effect of the structural analogues of GS and TA47 (daidzein and TA1) on insulin secretion.

In conclusion, the results from our experiments indicate that increased tyrosine phosphorylation *per se* is not required for glucose-stimulated insulin secretion, nor does an increase in the tyrosine phosphorylation state, as seen with PV, substantially stimulate insulin secretion. Islet PTKs are active under non-stimulatory conditions and our results suggest that they play a permissive role in the insulin secretory process by maintaining a regulatory protein(s) in a tyrosine-phosphorylated state. A likely candidate is a subunit of the VDCC, which would allow Ca^{2+} influx only when tyrosine phosphorylated. Although we do not at present have any direct evidence for the regulation of VDCCs by tyrosine phosphorylation, both α_1 and β VDCC subunits, which are expressed in islets (Ihara *et al.* 1995), may be regulated by protein phosphorylation by serine/threonine kinases (Campbell *et al.* 1988). In addition, the available protein sequence information for the β cell VDCC α subunit indicates that it contains several tyrosine residues with acidic residues at positions N-terminal to the tyrosine (Ihara *et al.* 1995) indicative of a PTK recognition site (Songyang & Cantley 1995).

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