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

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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 µM) increased tyrosine phosphorylation of several islet proteins. The PTK inhibitors genistein (GS) and tyrphostin A47 (TA47) inhibited islet tyrosine kinase activities and glucose-, 4α 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 Ca2+, 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 Ca2+ entry and it is proposed that they act at the site of the voltage-dependent Ca2+ channel which regulates Ca2+ 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 (Oberg et al. 1994). In addition, studies using neonatal islets (Sorensen 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 βTC3 cell line, that PTK inhibition causes inhibition of both tyrosine phosphorylation and insulin secretion in response to glucose and the cholinergic agonist carbamol (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 (Breleje 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, 4β phorbol myristate acetate (PMA), cyclic AMP and isobutyl methyloxanthine (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 [125I] for insulin iodination were from Amersham International (Amersham, Bucks, UK) and [γ32P]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 × 10⁶ 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 µm nitrocellulose membranes which were blocked overnight in phosphate buffered saline (PBS) supplemented with 0·05% (w/v) Tween 20 and 1% (w/v) BSA and then incubated overnight in

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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 Ca²⁺/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 HIIA and 50 µg/ml calmodulin, 0·5 mM CaCl₂, 1 mg/ml myosin light chains respectively) and 100 µM [γ³²P]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 [³²P]incorporation into histone HII (1 mg/ml) was determined in the presence of 96 µg/ml phosphatidylyserine, 6·4 µg/ml diolein and 1·3 mM CaCl₂ (Persaud & Jones 1995).

**Statistical analysis**

Results are presented, where appropriate, as means ± 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 µM), 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 µM 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 µM 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
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 ± 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Insulin secretion (% glucose-stimulated)</th>
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<tbody>
<tr>
<td>50 µM genistein</td>
<td>136 ± 7a</td>
</tr>
<tr>
<td>100 µM genistein</td>
<td>96 ± 12</td>
</tr>
<tr>
<td>200 µM genistein</td>
<td>34 ± 4b</td>
</tr>
<tr>
<td>500 µM genistein</td>
<td>3 ± 0.3b</td>
</tr>
<tr>
<td>500 µM daidzein</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>12·5 µM TA47</td>
<td>80 ± 6a</td>
</tr>
<tr>
<td>25 µM TA47</td>
<td>74 ± 3a</td>
</tr>
<tr>
<td>50 µM TA47</td>
<td>40 ± 4b</td>
</tr>
<tr>
<td>100 µM TA47</td>
<td>48 ± 6b</td>
</tr>
<tr>
<td>200 µM TA47</td>
<td>5 ± 1a</td>
</tr>
<tr>
<td>12·5 µM TA1</td>
<td>98 ± 8</td>
</tr>
<tr>
<td>25 µM TA1</td>
<td>141 ± 17a</td>
</tr>
<tr>
<td>50 µM TA1</td>
<td>143 ± 10a</td>
</tr>
</tbody>
</table>

*P*<0·05, **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 µM) caused a small, but significant, potentiation of glucose-stimulated secretion, but at concentrations of GS greater than 100 µM, secretion was significantly inhibited. The inactive analogue of GS, daidzein, was without effect on insulin release when used at a concentration (500 µM) 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 µM. The inactive analogue of TA47, TA1, did not inhibit secretion, but low concentrations (25 and 50 µM) significantly potentiated the response to glucose (Table 1). Pretreatment of islets with 100 µM 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 µM GS: 0·41 ± 0·08; +100 µM PV +500 µM GS: 0·55 ± 0·16; +200 µM TA47: 0·17 ± 0·05; +100 µM PV +200 µM 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 4α 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 µM GS: 0·31 ± 0·02; 10 mM KIC+200 µM 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 perifusion 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 µM 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 µM GS: 0·298 ± 0·007; +200 µM 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 µM tolbutamide: 628 ± 100% secretion at 5 mM glucose; +500 µM GS: 86 ± 14%; +200 µM TA47: 271 ± 51%; 10 µM glibenclamide: 528 ± 71% secretion at 5 mM glucose; +500 µM GS: 157 ± 37%; +200 µM TA47: 271 ± 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 glucose metabolites) and PTK inhibitors.
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 µM GS, 50 µM TA47) or maximally (500 µM GS, 200 µM TA47) inhibited insulin secretion. It can be seen from Fig. 4A that inclusion of 100 µM 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 µM GS (lane 4) or 200 µM 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 µM GS: 68 ± 4.2; +200 µM TA47: 51 ± 1.2, n=4), while TA47, but not GS, inhibited PKA activity (control: 70 ± 1.9 fmol/islet/min; +500 µM GS: 74 ± 0.9; +200 µM TA47: 32 ± 0.6, n=4). In both instances, the inhibitory effects of GS and TA47 were partial. In the CaMK assay, Ca²⁺ 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 µM GS, islet CaMK activity was not noticeably affected, but 200 µM 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 Ca²⁺ 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 Ca²⁺ 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.

**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
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 Ca²⁺, 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

### 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 Ca²⁺. Insulin secretion is expressed as pg/islet/h (± s.e.m.), and the numbers of observations are shown in parentheses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Insulin secretion (pg/islet/h)</th>
</tr>
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<tbody>
<tr>
<td>50 nM Ca²⁺</td>
<td>176 ± 12 (18)</td>
</tr>
<tr>
<td>10 µM Ca²⁺</td>
<td>490 ± 16* (18)</td>
</tr>
<tr>
<td>10 µM Ca²⁺ + 200 µM TA47</td>
<td>431 ± 35* (9)</td>
</tr>
<tr>
<td>10 µM Ca²⁺ + 500 µM GS</td>
<td>533 ± 32* (9)</td>
</tr>
<tr>
<td>500 µM cyclic AMP</td>
<td>442 ± 31* (18)</td>
</tr>
<tr>
<td>500 µM cyclic AMP + 200 µM TA47</td>
<td>501 ± 36* (9)</td>
</tr>
<tr>
<td>500 µM cyclic AMP + 500 µM GS</td>
<td>428 ± 26* (9)</td>
</tr>
<tr>
<td>500 nM PMA</td>
<td>517 ± 18* (18)</td>
</tr>
<tr>
<td>500 nM PMA + 200 µM TA47</td>
<td>478 ± 28* (9)</td>
</tr>
<tr>
<td>500 nM PMA + 500 µM GS</td>
<td>512 ± 34* (9)</td>
</tr>
</tbody>
</table>

*P<0.0001 versus secretion at 50 nM Ca²⁺, bP>0.1 versus appropriate control in absence of PTK inhibitor.
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 \(\beta\) cells from adult mouse islets (Jonas et al. 1995). The mode of action of GS on blockade of \(\beta\) 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 \(\alpha_1\) and \(\beta\) 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 \(\beta\) cell VDCC \(\alpha\) 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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