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Inhibition of glucose-stimulated insulin secretion by Ro 31-8220, a protein kinase C inhibitor

Shanta J. Persaud & Peter M. Jones

Biomedical Sciences Division, King's College London, Campden Hill Road, London W8 7AH, UK

The involvement of the family of protein kinase C (PKC) isoenzymes in the secretory response of rat islets of Langerhans to glucose, the major insulin secretagogue, was investigated using the PKC inhibitor Ro 31-8220, a derivative of staurosporine. Ro 31-8220 was a more selective PKC inhibitor than staurosporine in islets, having minimal effects on protein kinases activated by cyclic AMP or by Ca^{2+} and calmodulin. The secretory response to 4βPMA, an activator of phorbol ester-sensitive isoforms of PKC, was abolished by Ro 31-8220. Basal insulin secretion (2 mM glucose) was not affected by Ro 31-8220, but 20 mM glucose-induced insulin release was inhibited in a dose-dependent manner, maximally by ~50% at 10 μM Ro 31-8220. Higher concentrations of Ro 31-8220 (50 μM) did not further inhibit the secretory response to glucose and also caused ~50% inhibition of insulin secretion stimulated by 10 mM glyceraldehyde. Ca^{2+}-stimulated insulin secretion from electrically permeabilised islets was not inhibited by Ro 31-8220. Calphostin C, which inhibits some isoforms of PKC by interacting with the diacylglycerol binding site, unexpectedly caused a large (~10-fold) increase in secretion at 2 mM glucose, so could not be used in islets to further investigate the involvement of phorbol ester-sensitive PKC isoforms in the insulin secretory process. One possible explanation for our results using Ro 31-8220 is that phorbol ester-insensitive isoforms of PKC (ζ and/or ι) are involved in glucose-stimulated insulin secretion from rat islets.

Keywords: Islets of Langerhans; insulin secretion protein kinase C; phorbol esters

Introduction

Protein kinase C (PKC) was initially described as a Ca^{2+}- and phospholipid-dependent enzyme which is activated physiologically by diacylglycerol (DAG), and pharmacologically by tumour-promoting phorbol esters, which substitute for DAG (reviewed by Nishizuka, 1984). Early studies on the role played by PKC in pancreatic β-cell signal transduction mechanisms made use of 4β phorbol myristate (4βPMA) both as a short-term activator of the enzyme and as a means of depleting cellular content of PKC (down-regulation) by stimulating permanent activation and subsequent degradation (reviewed by Persaud *et al.*, 1992). However, elucidation of the involvement of this kinase in stimulus-secretion coupling in islets has become more complicated over the years with the discovery of new PKC isoforms, some of which do not bind DAGs or phorbol esters. Thus, in addition to the conventional, phorbol ester-sensitive α and β isoforms (Ito *et al.*, 1989; Onoda *et al.*, 1990), two phorbol ester-insensitive PKC isoforms (ζ and ι) have been identified recently in rat islets (Selbie *et al.*, 1993) and their existence means that earlier studies, which examined the secretory responses of islets/β-cell lines in which phorbol ester-sensitive isoforms of PKC had been down-regulated by prolonged exposure to 4βPMA, may not have provided a complete picture (e.g. Hii *et al.*, 1987; Metz, 1988; Persaud *et al.*, 1989a; Arkhammar *et al.*, 1989; Hughes *et al.*, 1990; Li *et al.*, 1990; Thams *et al.*, 1990). One alternative approach to down-regulation of PKC is the use of inhibitors which act at the ATP-binding site of the PKC catalytic domain and recent studies have indicated that one such potent inhibitor of PKC, staurosporine, inhibits glucose-stimulated insulin secretion (Easom *et al.*, 1989; Zawalich *et al.*, 1991; Persaud *et al.*, 1993). However, the non-selectivity of staurosporine as a PKC inhibitor in many cell types, including islets (Persaud *et al.*, 1993), makes it difficult to draw definite conclusions from experiments using this agent. A series of bisindolylmaleimide derivatives of staurosporine have been developed as potent, selective PKC inhibitors (Davis *et al.*, 1989), and in the past few years have been used in several cell types to investigate the role of PKC in signal transduction processes (e.g. Dieter & Fitzke, 1991; Nixon *et al.*, 1992; McKenna & Hanson, 1993; Walker & Watson, 1993). Studies of the effects of these inhibitors on purified PKC isoenzyme activity have revealed that they inhibit all isoforms tested (α, β_I, β_{II}, γ, ∈), with little selectivity for particular isoenzymes (Wilkinson *et al.*, 1993). We have now investigated the effects of one of these bisindolylmaleimides, Ro 31-8820, on the activity of PKC and other serine/threonine protein kinases in islets, and on glucose-induced insulin secretion, to examine whether PKC can be selectively inhibited in islets and whether this affects the secretory response to glucose.

Correspondence: Dr S.J. Persaud
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Results

Effect of Ro 31-8220 on islet PKC activity

Activity of PKC extracted from rat islets stimulated by Ca^{2+}, phosphatidylserine and diolein in the presence of 110 μM ATP was 303 ± 9.6 fmol/islet/minute ($n = 4$). 1 nM, 10 nM and 100 nM Ro 31-8220 did not affect islet PKC activity (107%, 101% and 107% maximal activity, respectively), but higher concentrations progressively inhibited PKC activity such that it was abolished by 10 μM Ro 31-8220 (Figure 1, left panel).

Effect of Ro 31-8220 on insulin secretion from intact islets

As expected, Ro 31-8220 inhibited insulin secretion stimulated by 4βPMA (2 mM glucose, 0.14 ± 0.02 ng/islet/h; 500 nM 4βPMA, 0.42 ± 0.05, $P < 0.001$ versus 2 mM glucose; 500 nM 4βPMA + 10 μM Ro 31-8220, 0.11 ± 0.01, $P < 0.001$ versus 500 nM 4βPMA, $n = 8–9$), but did not affect secretion in the presence of 2 mM glucose alone (107 ± 21% basal). Concentrations of Ro 31-8220 greater than 100 nM caused a significant inhibition of glucose-stimulated insulin secretion, with 10 μM Ro 31-8220 causing a 52 ± 5.8% inhibition of the secretory response to 20 mM glucose alone (Figure 1, right panel). Increasing the Ro 31-8220 concentration to 50 μM was marginally, but not significantly, more effective than 10 μM Ro 31-8220 (59 ± 10% inhibition, $P > 0.2$ versus 10 μM Ro 31-8220). 50 μM Ro 31-8220 also caused a significant, but incomplete inhibition of insulin secretion stimulated by glyceraldehyde (2 mM glucose, 0.10 ± 0.01 ng/islet/h; 10 mM glyceraldehyde, 2.84 ± 0.35, $P < 0.001$ versus

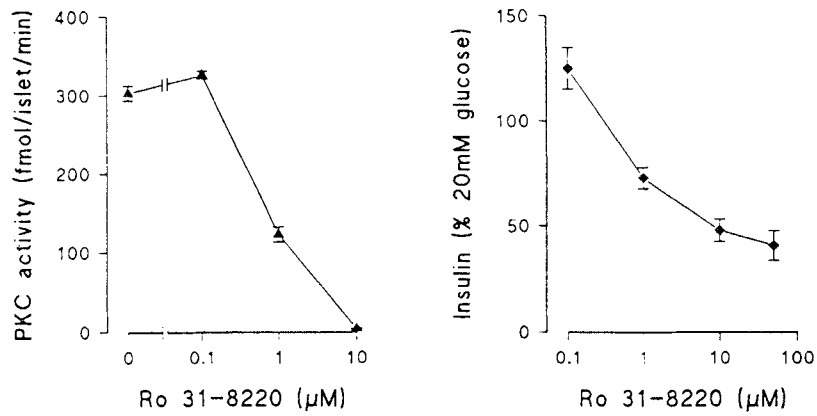

Figure 1 Effect of Ro 31-8220 on islet PKC activity and on glucose-induced insulin secretion. *Left panel:* Ro 31-8220 produced a dose-dependent inhibition of PKC purified from islets by ion-exchange chromatography. Total inhibition of islet PKC activity *in vitro* was achieved by 10 μM Ro 31-8220. Points show means ± SEM, $n = 3-4$. *Right panel:* Ro 31-8220 caused a dose-related inhibition of glucose-induced (20 mM) insulin release. Maximum inhibition was achieved by 10 μM Ro 31-8220 and represented an approximately 50% inhibition of glucose-induced insulin secretion. Points show means ± SEM, $n = 8-9$.

2 mM glucose; 10 mM glyceraldehyde + 50 μM Ro 31-8220, 1.41 ± 0.28, $P < 0.01$ versus 10 mM glyceraldehyde, $n = 8-9$). Insulin secretion stimulated by α-ketoisocaproic acid (KIC), the deamination product of leucine whose metabolism is confined to mitochondria, was also inhibited by Ro 31-8220 (2 mM glucose, 0.20 ± 0.04 ng/islet/h; 10 mM KIC, 1.99 ± 0.16, $P < 0.001$ versus 2 mM glucose; 10 mM KIC + 10 μM Ro 31-8220, 1.10 ± 0.14, $P < 0.001$ versus 10 mM KIC, $n = 9$).

Effect of Ro 31-8220 on islet PKA and CaMK activities

The effects of Ro 31-8220 on islet PKC activity and on glucose- and glyceraldehyde-induced insulin secretion were very similar to those observed for staurosporine, which we found to be a non-selective kinase inhibitor in rat islets (Persaud *et al.*, 1993). The activities of other islet serine/threonine protein kinases (PKA and CaMKs) were therefore measured in the presence of concentrations of Ro 31-8220 which had marked inhibitory effects on islet PKC activity (1 μM and 10 μM), to investigate whether Ro 31-8220 was acting as a selective PKC inhibitor in islets. PKA activity stimulated by cyclic AMP (in the presence of IBMX) was inhibited by only 5% by 1 μM Ro 31-8220, while a ten-fold higher concentration, which fully inhibited islet PKC activity (Figure 1, left panel), only caused a 32% inhibition of PKA activity (control, 783 ± 4 fmol/islet/min; +1 μM Ro 31-8220, 740 ± 6; +10 μM Ro 31-8220, 533 ± 2, $n = 4$). In contrast, 400 nM staurosporine, a concentration which maximally inhibits islet PKC activity *in vitro* (Persaud *et al.*, 1993), abolished islet PKA activity. In the presence of Ca^{2+} and calmodulin islet extracts phosphorylated exogenous myosin light chains, and also showed increased phosphorylation of several endogenous proteins (Figure 2). Neither 100 nM nor 1 μM Ro 31-8220 inhibited Ca^{2+} calmodulin-dependent protein phosphorylation, but 10 μM Ro 31-8220 caused a small inhibition of both exogenous and endogenous phosphorylations. In the presence of staurosporine (400 nM), islet CaMK activities were fully inhibited such that the phosphorylation profile did not differ significantly from that obtained in the absence of Ca^{2+} and calmodulin (Figure 2).

Effect of Ro 31-8220 on insulin secretion from electrically permeabilised islets

The inhibitory effect of Ro 31-8220 on islet CaMK activities *in vitro* was modest, but since CaMK may be involved in the secretory response to glucose (Wenham *et al.*, 1992, 1994) it could provide an explanation for the inhibition of glucose-induced insulin release by Ro 31-8220. The ability of Ro 31-8220 to inhibit Ca^{2+}-stimulated insulin secretion from electrically permeabilised islets was therefore examined. Both 4βPMA and Ca^{2+} stimulated insulin secretion from permeabilised islets (50 nM Ca^{2+}, 360 ± 60 pg/islet/h; 500 nM 4βPMA, 740 ± 113; 10 μM Ca^{2+}, 824 ± 92, $P < 0.01$ versus 50 nM Ca^{2+}, $n = 9$). As expected, the secretory response to 4β-PMA was inhibited by 10 μM Ro 31-8220 (442 ± 57 pg/islet/h, $P < 0.05$ versus 500 nM 4βPMA, $P > 0.2$ versus 50 nM Ca^{2+}, $n = 9$). Although 10 μM Ro 31-8220 inhibited glucose-induced insulin release from intact islets (Figure 1), it did not significantly affect Ca^{2+}-stimulated secretion from

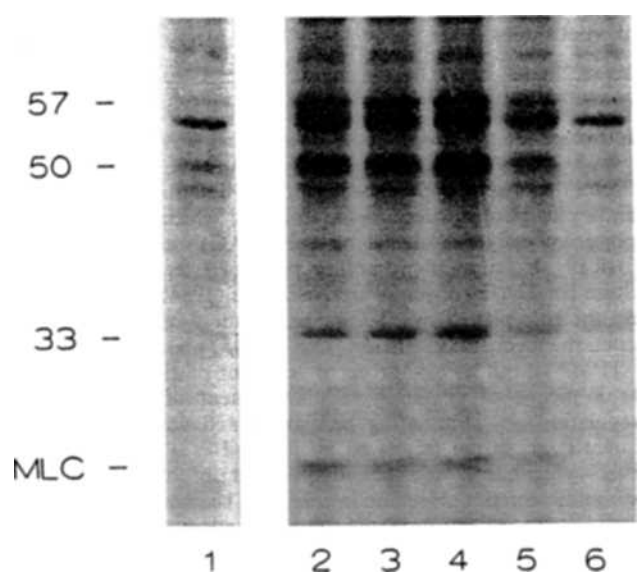

Figure 2 Effects of Ro 31-8220 and staurosporine on islet CaMK activities. Islet CaMK activities were not affected by Ro 31-8220 at concentrations of 100 nM and 1 μM, but were partially inhibited by 10 μM Ro 31-8220. 400 nM staurosporine abolished protein phosphorylation stimulated by Ca^{2+} and calmodulin. Numbers indicate molecular weights (kDa) of endogenous proteins which showed increased phosphorylation in the presence of Ca^{2+} and calmodulin, and MLC indicates the phosphorylation of exogenous myosin light chains. Lane 1: control (no Ca^{2+} or calmodulin). Lane 2: Ca^{2+}/calmodulin. Lane 3: Ca^{2+}/calmodulin + 100 nM Ro 31-8220. Lane 4: Ca^{2+}/calmodulin + 1 μM Ro 31-8220. Lane 5: Ca^{2+}/calmodulin + 10 μM Ro 31-8220. Lane 6: Ca^{2+}/calmodulin + 400 nM staurosporine

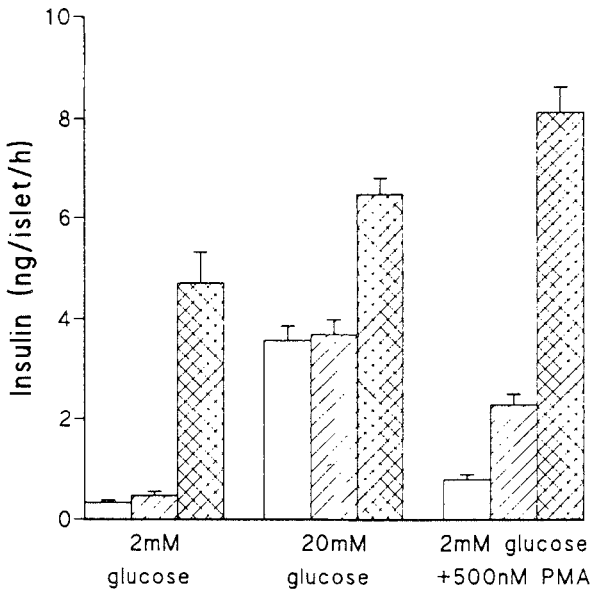

Figure 3 Effect of calphostin C on insulin secretion. Pre-exposure of islets to calphostin C (0.5 μM, hatched bars and 5 μM, cross-hatched bars) for one hour did not inhibit secretion in response to the PKC activator, 4βPMA (500 nM). In contrast, calphostin C had pronounced stimulatory effects on basal insulin secretion (2 mM glucose) and on secretory responses to 20 mM glucose or to 500 nM 4βPMA at 2 mM glucose. Bars show means + SEM, $n = 9$.

permeabilised islets (747 ± 104 pg/islet/h, $P > 0.2$ versus 10 μM Ca^{2+}, $P < 0.01$ versus 50 nM Ca^{2+}, $n = 9$).

Effect of calphostin C on 4βPMA- and glucose-stimulated insulin secretion

The inhibition of glucose-stimulated insulin secretion by Ro 31-8220 differed from our earlier results in which down-regulation of phorbol ester-sensitive isoforms of PKC did not affect the secretory response to glucose. Thus, in an attempt to distinguish between the contribution of phorbol ester-sensitive and -insensitive PKC isoforms to glucose-stimulated insulin release, islets were treated with calphostin C which is reported to exert inhibitory effects on PKC *in vitro* by acting at the DAG-binding site of the regulatory domain (Kobayashi *et al.*, 1989). This inhibitor therefore offers a potential means of discriminating between typical isoforms of PKC, which possess DAG-binding sites, and atypical isoforms, which do not, since it should not affect the activities of the β-cell PKC isozymes which are deficient in a DAG binding region (ζ and ι). When calphostin C was added to the islets at the same time as 20 mM glucose, it had no effect on insulin secretion, measured over 1 h (20 mM glucose, 6.24 ± 0.29 ng/islet/h; +calphostin C: 50 nM, 5.79 ± 0.50; 500 nM, 6.15 ± 0.73; 5 μM, 6.18 ± 0.56, $P > 0.2$ at all concentrations of calphostin C; $n = 7-9$). Surprisingly however, when islets were pre-incubated with 5 μM calphostin C for 1 h (37°C) prior to stimulation with 20 mM glucose, there was a marked (81 ± 4%) potentiation of secretion. This effect appeared to be totally unrelated to inhibition of PKC activity since the secretory response to 4βPMA, as well as that to 2 mM glucose alone, was also significantly enhanced by calphostin C (Figure 3).

Discussion

There is little doubt that the family of PKC isoenzymes plays important signalling roles in a diverse range of cell types, including islets of Langerhans. However, despite many attempts to pinpoint the precise role played by PKC in the secretory response of islets to nutrients, there is still no consensual view (see Persaud *et al.*, 1992). Early studies made use of the ability of phorbol esters to permanently activate PKC, ultimately causing its degradation. Such studies in islets showed that Ca^{2+}/phosphatidylserine/diolein-sensitive PKC activity was negligible after overnight exposure of islets to 4βPMA, that the islets no longer showed an acute secretory response to 4βPMA, but that they were still responsive to both glucose and glyceraldehyde (Hii *et al.*, 1987; Persaud *et al.*, 1989a, 1991). We concluded from these experiments that phorbol ester-sensitive isoforms of PKC expressed in β cells (α, β and possibly δ and ∈) were unlikely to be essential for glucose-induced insulin secretion, but we did not rule out a possible role for 4βPMA-insensitive isoforms of the kinase which were not down-regulated by phorbol ester treatment of islets. The discovery of novel isoforms of PKC which have only one cysteine-rich repeat of amino acids in the C1 region of the regulatory domain, and which therefore do not bind phorbol esters or DAGs (reviewed by Hug & Sarre, 1993), revealed that definitive conclusions on the role played by PKC in nutrient-induced insulin secretion could not be obtained from 'PKC down-regulation' experiments. However, the existence of phorbol ester-insensitive PKC isoforms does not detract from the conclusion that isozymes which are depleted by phorbol ester treatment are not required for secretory responses to nutrients, and this has been confirmed by an alternative approach, that of antisense oligonucleotide depletion of the α and β isoforms of PKC in islets (Persaud & Jones 1993).

How then, can these earlier results be reconciled with the current results? Two phorbol ester-insensitive PKC isoforms (ζ and ι) are known to be expressed in rat islets (Wetsel *et al.*, 1992; Selbie *et al.*, 1993), and while these will not be depleted by prolonged exposure to 4βPMA they are likely to be inhibited by Ro 31-8220, since it exerts its action at the ATP-binding site of kinases. At present no information is available on the sensitivity of ζ or ι isoforms to inhibition by Ro 31-8220, but experiments which examined the inhibitory action of a series of bisindolylmaleimides, including Ro 31-8220, on the activities of a range of PKC isoforms indicated that all isoforms tested (α, β_I, β_{II}, γ, ∈) were susceptible to inhibition by the Roche compounds (Wilkinson *et al.*, 1993). The most likely explanation for the inhibitory effects of Ro 31-8220 on glucose-stimulated insulin secretion is that it is inhibiting the activities of phorbol ester-insensitive PKC isoforms (ζ, ι and/or other atypical isoforms not yet identified in islets such as λ or μ; Johannes *et al.*, 1994) which are required for a full secretory response to nutrients. Whether this is an appropriate explanation depends largely on whether Ro 31-8220 truly is inhibiting insulin secretion through the inhibition of PKC isoform(s) or whether it has non-selective effects in islets.

Since the secretory response to glucose is dependent on its metabolism within β cells, one possible mode of action of Ro 31-8220 could be through inhibition of β cell glucose handling. There are several reasons why this is unlikely to be the case. First, the Ro 31-8220 parent compound, staurosporine, had no effect on glucose metabolism at concentrations which inhibited glucose-stimulated insulin secretion (Zawalich *et al.*, 1991). Second, inhibition of glucose metabolism would be expected to fully inhibit glucose-induced insulin secretion, but the inhibitory effect of Ro 31-8220 was only partial, even at very high concentrations. Third, the ability of Ro 31-8220 to inhibit insulin release stimulated by both glyceraldehyde and KIC suggests that its effects are distal to glucose phosphorylation and glycolytic metabolism.

Our experiments have revealed that Ro 31-8220 is a more effective inhibitor of islet PKC than of PKA or CaMK activities. However, at high concentrations (10 μM) it does exert inhibitory effects on islet PKA and CaMK activities *in vitro*. The small (~30%) inhibition of PKA activity by 10 μM Ro 31-8220 is unlikely to account for the inhibitory effects of Ro 31-8220 on the secretory response to glucose since our

earlier studies have indicated that complete inhibition of islet PKA activity does not significantly inhibit glucose-stimulated insulin secretion (Persaud *et al.*, 1990). It is conceivable that the capacity of Ro 31-8220 to inhibit CaMK is related to its inhibitory effect on secretion since CaMKII has been implicated in glucose-stimulated insulin release (Harrison *et al.*, 1986; Wenham *et al.*, 1992, 1994; Niki *et al.*, 1993). However, the secretory response to 10 μM Ca^{2+} in electrically permeabilised islets was not affected by a concentration of Ro 31-8220 which inhibited CaMK activities *in vitro* suggesting that the inhibition of CaMK is not responsible for the inhibition of stimulated secretion. Since the slight inhibitory effects of Ro 31-8220 on islet PKA and CaMK activities do not appear to mediate the inhibition of nutrient-induced secretion, our data would tend to support the involvement of PKCζ and/or PKCι (or other atypical PKC isoforms) in β-cell secretory responses to glucose, glyceraldehyde and KIC.

Another approach to determining the relative importance of typical and atypical PKC isozymes in the insulin secretory process was the use of calphostin C, a microbial compound which inhibits PKC by inhibiting binding of phorbol esters (and DAGs) to the regulatory domain of PKC (Kobayashi *et al.*, 1989). Calphostin C should selectively inhibit the phorbol ester-sensitive isoforms of PKC without affecting the activity of the ζ and ι isoforms since they do not contain a phorbol ester-binding site. However, in spite of its reported usefulness as a PKC inhibitor in other cell types (Tao *et al.*, 1992; Shimamoto *et al.*, 1993), calphostin C stimulated, rather than inhibited, 4βPMA-induced secretion. The secretory responses at substimulatory (2 mM) and maximal stimulatory (20 mM) concentrations of glucose were also increased by pre-exposure of islets to calphostin C indicating that it exerts generalised stimulatory effects on secretion. PKC-independent effects of calphostin C, including inhibition of mitochondrial electron transport (Berridge & Tan, 1992), have been reported previously and these obviously limit its usefulness as a tool for dissecting out the roles played by phorbol ester-sensitive and -insensitive PKC isoforms.

In summary, the results of the present study indicate that Ro 31-8220 is a fairly selective inhibitor of PKC in islets and its use has revealed that phorbol ester-insensitive isoforms may play a role in the stimulation of insulin secretion by glucose. The intracellular mechanisms by which glucose could activate the atypical PKC isoforms in islets is not clear, but one possible candidate is phosphatidylinositol 3,4,5-trisphosphate which has been shown to activate PKCζ *in vitro* (Nakanishi *et al.*, 1993). Alternatively, if β-cell atypical PKC isoforms exhibit constitutive, activator-independent activity, as in other cell types (reviewed by Hug & Sarre, 1993), they need not be activated by glucose (or some consequence of its metabolism) *per se*, but inhibition of their activities may result in a compromised secretory response to glucose. It should be emphasised that if these isoforms of PKC are involved in nutrient-stimulated insulin secretion, they are not responsible for the full secretory response since Ro 31-8220 maximally inhibited secretion by only ~50%. It is not yet clear which signal transduction system(s) is responsible for the component of the glucose-induced secretory response which is insensitive to inhibition by Ro 31-8220, but it seems likely that protein kinases are not involved since staurosporine, a non-selective kinase inhibitor, also only partially inhibited nutrient-induced insulin secretion (Easom *et al.*, 1989; Persaud *et al.*, 1993).

Materials and methods

Collagenase (type XI), BSA (fraction V), 4β phorbol myristate acetate (4βPMA), cyclic AMP, isobutyl methylxanthine (IBMX), calmodulin, myosin light chains, staurosporine, phenylmethylsulphonyl fluoride (PMSF), leupeptin, diolcin, phosphatidylserine, histone (types IIIs and IIa) and antibiotics were purchased from Sigma Chemical Co. (Dorset, UK). Diethylaminoethyl (DEAE) cellulose and glass microfibre filters (GF/C) were obtained from Whatman International Ltd. (Kent, UK). Na[^{125}I] for insulin iodination was from Amersham International (Buckinghamshire, UK) and [γ^{32}P]ATP (3000 Ci/mmol) was from DuPont (UK) Ltd (Hertfordshire, UK). Ro 31-8220 was kindly provided by Dr Geoff Lawton, Roche Research Centre (Hertfordshire, UK). Calphostin C was purchased from Calbiochem-Novabiochem (UK) Ltd. (Nottingham, UK). All other reagents were of analytical grade from BDH (Dorset, UK). Rats (Sprague Dawley; 150–200 g) were supplied by King's College London Animal Unit.

Islet isolation and permeabilisation

Islets of Langerhans were isolated from rat pancreata by collagenase digestion (Jones *et al.*, 1993). In some experiments islets were permeabilised by exposure to an electric field (3.4 kV/cm, 4°C) in a 'permeation' buffer containing 140 mM potassium glutamate. 7 mM $MgSO_4$, 5 mM ATP, 5 mM glucose, 1 mM EGTA, 15 mM HEPES and 0.5 mg/ml BSA, with $CaCl_2$ added to give a Ca^{2+} concentration of 50 nM (Jones *et al.*, 1985).

Insulin secretion

Groups of three intact islets were incubated at 37°C for 1 h in 600 μl of a bicarbonate-buffered (pH 7.4) physiological salt solution (Gey & Gey, 1936) supplemented with 2 mM glucose, 2 mM $CaCl_2$, 0.5 mg/ml BSA and test substances of interest. In some experiments, islets were preincubated with calphostin C (1 h, 37°C) before the addition of agonists. For secretion experiments using electrically permeabilised islets, groups of five islets were incubated at 37°C for 1 h in 500 μl of permeation buffer supplemented with 500 nM 4βPMA or 10 μM Ca^{2+}, in the absence or presence of Ro 31-8220. Insulin secreted into the supernatant from both intact and permeabilised islets was determined by radioimmunoassay (Jones *et al.*, 1988).

Protein kinase C purification and assay

PKC was partially purified from freshly isolated islets by a modification of a previously described method (Persaud *et al.*, 1989b). Briefly, the islets were resuspended in 200 μl of buffer A (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 sonicated (MSE Soniprobe, 3 × 15 s, 6 μ, 4°C). PKC activity of the islet sonicate was solubilised by incubation in the presence of 1% (v/v) nonidet-P40 (30 min, 4°C) and the extract was applied to DEAE-cellulose resin (~200 μl packed volume) in a 1.5 ml centrifuge tube and gently mixed. After brief centrifugation (6000 g, 15 s), the supernatant was discarded, the resin was washed three times with 1 ml buffer A, and PKC was recovered with buffer A supplemented with 120 mM NaCl. Activity of islet Ca^{2+}- and DAG-sensitive PKC isoforms was assayed by measuring the transfer of ^{32}P from [γ^{32}P]ATP to histone type IIIs, in the presence of 1.3 mM $CaCl_2$, 96 μg/ml phosphatidylserine and 6.4 μg/ml diolein, essentially as described (Persaud *et al.*, 1989b).

Protein kinase A assay

Protein kinase A (PKA) activity of islet extracts was measured by a modification of the PKC assay. Islets were sonicated on ice in buffer A (3 × 15 s) and the extract was used directly to assay PKA activity by measuring the incorporation of ^{32}P, from [γ^{32}P]ATP, into histone type IIa. 10 μl of islet extract (equivalent to 20 islets) were incubated (10 min, 30°C) in the presence of 100 μM cyclic AMP, 100 μM IBMX, 11.1 mM $MgAc_2$, 1.1 mg/ml histone IIa and 110 μM

[γ^{32}P]ATP (specific radioactivity 0.3 Ci/mmol), in a final assay volume of 30 µl. The reaction was stopped by the addition of 1 ml TCA (10% w/v) and 50 µl BSA (10 mg/ml) and phosphorylated histone was separated from unincorporated [γ^{32}P]ATP by filtration onto GF/C filters. Radioactivity incorporated into histone was determined by scintillation counting.

Ca^{2+}/calmodulin-dependent kinase assay

Activities of islet Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) were determined by measuring Ca^{2+}/calmodulin-dependent phosphorylation of endogenous and exogenous substrates by islet extracts. Islets were disrupted in buffer A by sonication (3 × 15 s, 4°C) and 10 µl of extract (equivalent to 60 islets) were incubated (10 min, 30°C) in the presence of 50 µg/ml calmodulin, 0.5 mM $CaCl_2$, 11.1 mM $MgAc_2$, 1 mg/ml myosin light chains and 100 µM [γ^{32}P]ATP (specific radioactivity 1.8 Ci/mmol), in a final assay volume of 30 µl. The reaction was terminated by addition of 30 µl of a buffer containing 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.2% (w/v) bromophenol blue in 125 mM Tris/HCl, pH 6.8 and the samples were boiled for 3 min. ^{32}P incorporation into myosin light chains and endogenous islet proteins was determined by fractionation of proteins on 10% polyacrylamide gels in the presence of SDS, followed by autoradiography. Molecular weights of phosphorylated proteins were determined from a calibration curve constructed from the migration positions of standard proteins of known molecular weights.

Statistical analyses

Results are expressed as means ± SEM. Differences between means were analysed by Student's t tests and considered significant when $P < 0.05$.

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