Effects of a phorbol ester and clomiphene on protein phosphorylation and insulin secretion in rat pancreatic islets

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The potentiation of glucose-stimulated insulin release induced by 100 nM-12-*O*-tetradecanoylphorbol 13-acetate (TPA) was inhibited by clomiphene, an inhibitor of protein kinase C (PK C), in a dose-dependent manner. Clomiphene at concentrations up to 50 μ M had a modest inhibitory action (27 %) on insulin release stimulated by 10 mM-glucose alone, but had no effect on the potentiation of insulin release induced by forskolin. Islet PK C activity, associated with a particulate fraction, was stimulated maximally by 100 nM-TPA. This stimulation was blocked by clomiphene in a dose-dependent manner, with 50 % inhibition at 30 μ M. Incubation of intact islets with TPA after preincubation with [32 P] P_i and 10 mM-glucose to label intracellular ATP resulted primarily in enhanced phosphorylation of a 37 kDa protein (mean value, $\pm$ S.E.M., 36700 ± 600 Da; $n = 7$). This increased phosphorylation was blocked by the simultaneous inclusion of clomiphene. Subcellular fractionation revealed the presence of the 37 kDa phosphoprotein in a 24000 g particulate fraction of islet homogenates. Neither clomiphene nor TPA affected the rate of glucose oxidation by islets. These results show that the phosphorylation state of a 37 kDa membrane protein parallels the modulation of insulin release induced by TPA and clomiphene and support a role for PK C in the insulin-secretory mechanism.

INTRODUCTION

Stimulation of insulin secretion by many agents, including glucose, is accompanied by enhanced turnover of inositol phospholipids (Best & Malaisse, 1984; Dunlop & Larkins, 1984). Hydrolysis of phosphatidylinositol biphosphate produces two second messengers; inositol triphosphate, which can mobilize intracellular Ca^{2+} in islets of Langerhans (Wolf *et al.*, 1985), and diacylglycerol, which activates islet protein kinase C (PK C) (Tanigawa *et al.*, 1982; Lord & Ashcroft, 1984). The tumour-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which activates PK C by substituting for diacylglycerol (Castagna *et al.*, 1982), is a potent stimulator of insulin release (Virji *et al.*, 1978; Jones *et al.*, 1985; Tamagawa *et al.*, 1985; Hii *et al.*, 1986; Thams *et al.*, 1986; Hughes *et al.*, 1987), suggesting a physiological role for PK C in the secretory mechanism. PK C has been shown to phosphorylate endogenous protein substrates in insulin-producing tissues (Thams *et al.*, 1984; Harrison *et al.*, 1984; Brocklehurst & Hutton, 1984), although the role of these protein substrates in the insulin-secretory mechanism is unclear. The non-steroidal anti-oestrogen clomiphene (a triphenylethylene compound) is a potent inhibitor of PK C (Huai-de Su *et al.*, 1985; O'Brian *et al.*, 1986), and in the present study we have used clomiphene and TPA to study the role of PK C in the insulin-secretory mechanism. We have investigated the effects of these two agents on insulin release, PK C activity and protein phosphorylation in rat islets in order to correlate changes in PK C activity with modulation of insulin release.

MATERIALS AND METHODS

Materials

AcrylAide was from Miles Laboratories, Slough, Bucks., U.K. Forskolin was from Calbiochem, Cambridge BioScience, Cambridge, U.K. TPA was from P-L Laboratories, Northampton, Northants., U.K. Guinea-pig anti-insulin serum was from Wellcome Reagents, Beckenham, Kent, U.K. Rat insulin standard was a gift from Dr. A. J. Moody, Novo Research Laboratories, Copenhagen, Denmark. ^{125}I -insulin, [U - ^{14}C]glucose and [^{32}P] P_i were from Amersham International, Amersham, Bucks., U.K. [γ - ^{32}P]ATP was from New England Nuclear, Dreiech, W. Germany. Clomiphene citrate, diolein, dithiothreitol, BSA, histone H1, phosphatidylserine, PMSF and standard proteins for M_r determination were from Sigma Chemical Co., Poole, Dorset, U.K. X-OMAT AR film was from Kodak, Hemel Hempstead, Herts., U.K.

Isolation of islets of Langerhans

Islets of Langerhans were prepared by collagenase digestion of pancreases from male Wistar rats (Sutton *et al.*, 1986). Free islets were collected under a dissecting microscope with a wire loop.

Insulin release

In insulin-release experiments, batches of five islets were incubated for 2 h at 37 °C in a modified Krebs bicarbonate medium containing 20 mM-Hepes, pH 7.4, and 2 mg of BSA/ml (Christie & Ashcroft, 1985).

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; BSA, bovine serum albumin; PK C, protein kinase C; PMSF, phenylmethanesulphonyl fluoride.

Portions of incubation medium were collected and stored at -20°C in phosphate buffer containing BSA and merthiolate until assayed by radioimmunoassay using a charcoal separation method (Ashcroft & Crossley, 1975).

Glucose oxidation

Glucose oxidation was measured in freshly isolated islets incubated for 2 h as described previously (Ashcroft *et al.*, 1970).

Subcellular fractionation

Islets were fractionated by differential centrifugation essentially as described by Christie & Ashcroft (1985). Briefly, islets were homogenized (20 strokes of a motor-drive Teflon homogenizer) in ice-cold 0.3 M-sucrose/50 mM-sodium phosphate (pH 7)/1 mM-PMSF/2 mM-benzamidine. When phosphorylation of endogenous islet proteins was studied (see below), the homogenization medium also contained 50 mM-NaF, 2 mM-EDTA and 0.2 mM-EGTA in order to inhibit endogenous protein kinases and phosphatases. The protein content of fractions was measured as described by Bradford (1976), with BSA as standard.

PK C assay

PK C activity was assayed as described by Lord & Ashcroft (1984). The standard assay mixture (50 μl) contained 1 μmol of Tris/HCl (pH 7.4), 0.25 μmol of MgCl_2 , 10 μg of histone H1, 0.8 μg of phosphatidylserine, 0.25 nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 1 nmol of ATP, 25 nmol of EGTA, 24 nmol of CaCl_2 and either 60 ng of diolein or 0.2–20 pmol of TPA. The free Ca^{2+} concentration was calculated (Severson *et al.*, 1974) to be 5 μM . Reactions were initiated by addition of samples of islet fractions containing 2–5 μg of protein. Reactions were terminated after 3 min by pipetting 40 μl samples of the incubation mixture on to paper squares saturated with ATP, which were immediately immersed in ice-cold 5% trichloroacetic acid/0.25% sodium tungstate solution. The paper squares were washed twice in excess 5% trichloroacetic acid/0.25% sodium tungstate solution and then in excess methylated spirit, dried, and counted for radioactivity in toluene scintillant in a Packard liquid-scintillation counter (Tri-Carb 4000 series). PK C activity was calculated from the difference between radioactivity (c.p.m.) bound in the presence and in the absence of Ca^{2+} /phosphatidylserine/diolein or TPA.

Phosphorylation of peptides in intact islets

Batches of 80–100 islets were preincubated in 0.15 ml of the Hepes-buffered Krebs medium containing 10 mM-glucose, BSA (2 mg/ml) and 0.1 mCi of $[\text{}^{32}\text{P}]\text{P}_i$ (carrier-free) for 1 h at 37°C . TPA and clomiphene, diluted from stock solutions of 2 mM and 100 mM in dimethyl sulfoxide to final concentrations of 100 nM and 50 μM respectively, were added in 50 μl of Hepes-buffered Krebs medium and the incubations continued for a further 30 min. Controls received Hepes-buffered Krebs medium containing the appropriate concentration of dimethyl sulfoxide only. At the end of the incubation period, the islets were washed twice by centrifugation in 1 ml of Hepes-buffered Krebs medium containing 10 mM-glucose and the test agent where appropriate. The final islet pellet was either homogenized and fractionated by differential centrifugation as described above or

sonicated in 50 μl of ice-cold 50 mM-sodium phosphate (pH 7)/50 mM-NaF/2 mM-EDTA/0.2 mM-EGTA/0.5 mM-PMSF/2 mM-benzamidine. SDS, Bromophenol Blue and mercaptoethanol were added at final concentrations of 1%, 0.01% and 5% (w/v) respectively, and the samples were boiled for 5 min. The phosphopeptides were analysed by SDS/polyacrylamide-gel electrophoresis and quantified by autoradiography using a Joyce-Loebl densitometric scanner as described previously (Christie & Ashcroft, 1985).

RESULTS

Effects of TPA and clomiphene on glucose-stimulated insulin release and glucose oxidation in rat islets

Insulin release was stimulated approx. 8-fold on raising the glucose concentration in the medium from 2 to 10 mM. Glucose-stimulated insulin release was potentiated further by 40 and 68% when the incubation medium contained TPA (100 nM) or forskolin (10 μM) respectively (Table 1). The presence of 50 μM -clomiphene had a modest inhibitory effect (27%) on glucose-stimulated insulin release, whereas the inhibition of TPA-stimulated insulin release was much more pronounced. In contrast, the addition of clomiphene to the incubation medium had no effect on the potentiation of glucose-induced insulin release by forskolin. Table 1 also shows the effects of the various agents on glucose oxidation in parallel incubations. The presence of TPA or clomiphene added alone or collectively had no significant effect on islet glucose oxidation in the presence of 10 mM-glucose. A more detailed study of the inhibitory action of clomiphene on glucose-stimulated insulin release in the presence and absence of TPA (100 nM) is shown in Fig. 1. Clomiphene had only a modest inhibitory effect on glucose-stimulated insulin release at concentrations up to 50 μM , although, when the concentration in the incubation medium was further increased to 100 μM , insulin secretion was inhibited by 64%. Potentiation of glucose-stimulated insulin release by TPA was much more sensitive to the action of clomiphene (Fig. 1); the increment in insulin release induced by the phorbol ester was completely blocked by the inclusion of 50 μM -clomiphene in the incubation medium, and higher concentrations of clomiphene (100 μM) inhibited the total secretory response by 70%.

Effect of clomiphene on islet PK C activity stimulated by TPA

The subcellular location of PK C was dependent on the homogenization conditions. When 1 mM-EGTA was included in the homogenization buffer, the activity appeared predominantly (> 90%) in the supernatant fraction after centrifugation at 24000 g for 30 min. In the absence of EGTA, and in the presence therefore of trace amounts of Ca^{2+} , PK C was recovered mainly (> 75%) in the 24000 g particulate fraction. Since activation of the enzyme in intact tissues is accompanied by translocation from a cytosolic to a membrane location (Kraft & Anderson, 1983; Drust & Martin, 1985; Wooten & Wrenn, 1984; Hirota *et al.*, 1985), we elected to prepare islet homogenates in the absence of EGTA for measurement of PK C activity.

Fig. 2(a) shows the stimulation of PK C activity by TPA in a 24000 g particulate fraction of islet homo-

Table 1. Insulin release and glucose oxidation in rat islets

Batches of five islets were incubated at 37 °C for 2 h in Hepes-buffered Krebs medium containing BSA (2 mg/ml). Insulin released into the medium was measured by radioimmunoassay. For measurement of glucose oxidation the medium contained [U-¹⁴C]glucose, and the ¹⁴CO₂ evolved was absorbed in Hyamine and counted by liquid-scintillation spectrometry. Data are given as means ± S.E.M. for the numbers of observations in parentheses. The significance of the effects of clomiphene and TPA were assessed by Student's *t* test: ^a*P* < 0.001 versus 10 mM-glucose; ^b*P* < 0.01 versus 10 mM-glucose; ^c*P* < 0.001 versus 10 mM-glucose + TPA; NS, not significant versus 10 mM-glucose.

Incubation conditions			Insulin release (μunits/h per islet)	Glucose oxidation (pmol/h per islet)
Glucose (mM)	Clomiphene (μM)	Other additions		
2	—	—	35 ± 11 (15)	—
10	—	—	270 ± 18 (15)	22.0 ± 2.2 (9)
10	50	—	197 ± 16 (15) ^b	23.3 ± 1.7 (10) NS
10	—	100 nM-TPA	372 ± 9 (10) ^a	26.7 ± 1.8 (9) NS
10	50	100 nM-TPA	187 ± 10 (10) ^c	27.7 ± 3.4 (9) NS
10	—	10 μM-forskolin	454 ± 21 (15) ^a	—
10	50	10 μM-forskolin	439 ± 32 (15) ^a	—

Fig. 1. Inhibition by clomiphene of glucose-stimulated insulin release in the presence and the absence of TPA

Batches of five islets were incubated for 2 h at 37 °C in Hepes-buffered Krebs medium containing BSA (2 mg/ml) and 10 mM-glucose in the absence (■) or the presence (◇) of TPA (100 nM) at different clomiphene concentrations. In order to combine data from several experiments, results are expressed relative to the control rate of insulin release seen in the presence of 10 mM-glucose (272 ± 22 μunits/h per islet) in the same experiment and are given as means ± S.E.M. for 15 observations.

genates. Maximal PK C activity was obtained when the incubation medium contained 100 nM-TPA. The dose-dependent inhibition of TPA-stimulated PK C activity by clomiphene is shown in Fig. 2(b). Maximal inhibition of PK C was obtained when the incubation medium contained 100 μM-clomiphene; the concentration giving a half-maximal effect (IC_{50}) was 30 μM.

Effect of TPA and clomiphene on protein phosphorylation in intact islets

Stimulation of intact islets by 100 nM-TPA, after preincubation with 10 mM-glucose and [³²P]P_i to label intracellular ATP, resulted in enhanced phosphorylation of a number of peptide bands. The most marked and consistent effect was seen for a 37 kDa peptide (its mean

value, ± S.E.M. for seven experiments, was 36700 ± 600 Da). Fig. 3(a) shows a densitometric trace of an autoradiogram after separation of islet proteins by SDS/polyacrylamide-gel electrophoresis from a typical experiment. For each set of densitometric traces in an experiment, a baseline was interpolated and peak heights were measured. When expressed relative to controls in the same experiment, the mean peak height of the 37-kDa band was increased to $172 \pm 20\%$ (mean ± S.E.M.; *n* = 7) by TPA. The difference was significant (*P* < 0.01) by a paired *t* test. In three experiments the effect of clomiphene (50 μM) on TPA-stimulated phosphorylation of the 37 kDa peptide was studied. Fig. 3(b) shows the results of a representative experiment. The presence of clomiphene significantly (*P* < 0.02, paired *t* test) decreased the stimulatory effect of TPA on the 37 kDa peptide to a value ($116 \pm 10\%$) not significantly greater than control.

After removal of cell debris and nuclei by centrifugation at 600 *g* for 10 min, the 37 kDa peptide could be recovered in a 24000 *g* particulate fraction (Fig. 3c).

DISCUSSION

PK C has been detected in extracts of rat (Lord & Ashcroft, 1984) and mouse (Thams *et al.*, 1984) islets of Langerhans, and its presence in β-cells was confirmed by purification from the cloned β-cell line HIT-T15 (Lord & Ashcroft, 1984). Evidence that PK C may play a role in the regulation of insulin secretion has been sought by comparing effects of various modulators of PK C on insulin release. Tumour-promoting phorbol esters, which are thought to substitute for diacylglycerol as activators of PK C (Castagna *et al.*, 1982), have been shown to stimulate insulin secretion from isolated islets (Virji *et al.*, 1978; Malaisse *et al.*, 1980; Harrison *et al.*, 1984; Hii *et al.*, 1986; Hughes *et al.*, 1987), from rat insulinoma (Hutton *et al.*, 1984), and from HIT T15 β-cells (Ashcroft *et al.*, 1986). In either rat (Hughes *et al.*, 1987) or mouse (Thams *et al.*, 1986) islets, the effect of TPA has been shown to be that of a potentiator, i.e. no effect is seen in the absence of glucose. Potentiation of glucose-stimulated insulin release by oleoylacetylgllycerol, a synthetic diacylglycerol which activates PK C, has also been observed

Fig. 2. Effects of TPA and clomiphene on islet PK C activity

PK C activity was assayed in a particulate membrane fraction prepared from islet homogenates. Membrane protein (5 μ g) was incubated in the presence of 5 μ M- Ca^{2+} , 0.8 μ g of phosphatidylserine and either (a) various concentrations of TPA or (b) with TPA (100 nM) and various concentrations of clomiphene.

(Malaisse *et al.*, 1985). Several inhibitors of PK C have been reported to inhibit insulin secretion, including retinal (Harrison *et al.*, 1984), chlorpromazine (Tanigawa *et al.*, 1982), polyamines, such as spermine (Thams *et al.*, 1986) and polymyxin B (Stutchfield *et al.*, 1986), and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7) (Malaisse & Sener, 1985). Several previous studies have demonstrated that endogenous protein substrates for PK C can be found in extracts of rat (Harrison *et al.*, 1984) or mouse (Thams *et al.*, 1984) islets or rat insulinoma (Brocklehurst & Hutton, 1984). However, a limitation of previous inhibitor studies has been that protein phosphorylation has not been measured in parallel with insulin secretion to confirm that the inhibitors are indeed acting on PK C in the intact islet. The main aim of the present study was to investigate in intact islets whether changes in phosphorylation of specific islet proteins by PK C could be correlated with changes in insulin secretion rate, and we have used TPA and clomiphene as an activator and an inhibitor of PK C respectively for this purpose.

Clomiphene proved to be a potent inhibitor of islet PK C activity at similar concentrations to those reported for inhibition of protein kinase C from pig or rat brain (Huai-de Su *et al.*, 1985; O'Brian *et al.*, 1986). At concentrations up to 50 μ M, clomiphene inhibited the insulin-secretory response associated with TPA stimulation, but had only a modest inhibitory action on glucose-stimulated insulin release. In addition, 50 μ M-clomiphene had no effect on glucose oxidation in rat islets, nor did it inhibit the potentiation of glucose-stimulated insulin release induced by forskolin, an activator of cyclic AMP-dependent protein kinase. It has previously been reported that another triphenylethylene compound, tamoxifen, which blocks PK C activity, exerts no inhibitory action against cyclic AMP-dependent protein kinase from pig heart (Huai-de Su *et al.*, 1985). Our data suggest that clomiphene may selectively inhibit PK C and hence may prove a useful tool in the study of the mechanism of insulin release.

The identity and role of the substrates of PK C in the insulin-secretory mechanism are unknown. In our study, the main substrate for PK C in intact islets was a 24000 g-particulate-fraction protein of 37 kDa. Changes in the phosphorylation state of this peptide in intact islets induced by TPA and clomiphene paralleled the modulation of insulin release induced by these two agents. These data clearly implicate the phosphorylation of the 37 kDa peptide by PK C in the insulin-secretory process. Dunlop

& Larkins (1986), using an experimental system similar to our own, reported that stimulation of cultured neonatal islets with high concentrations of TPA (2 μ M) resulted in phosphorylation of a particulate protein of 40 kDa. Interestingly, a peptide of similar molecular size also showed enhanced phosphorylation in response to stimulation by glucose.

Thams *et al.* (1984) adopted a different methodology for investigating the substrates of PK C in mouse pancreatic islets. They prepared subcellular fractions and used [32 P]ATP as a substrate for PK C. They reported that only cytosolic proteins and not membrane-associated peptides became phosphorylated by endogenous PK C activity. However, we have found that when subcellular fractions are prepared from islets under similar conditions to those used by Thams *et al.* (1984) (i.e. in the presence of 1.0 mM-EGTA), membrane-bound PK C activity dissociates from the particulate fraction and is found in the cytosol. In another study of relevance to the present one, Turgeon & Cooper (1986) showed that PK C activity associated with secretory granules from sheep anterior pituitary glands phosphorylated a granule protein of 36 kDa on stimulation by Ca^{2+} and phospholipid. A 29 kDa phosphopeptide substrate for PK C in rat insulinoma has also been localized to secretory granules (Brocklehurst & Hutton, 1984).

The present and previous results therefore converge in suggesting that activation of islet PK C leads to enhanced phosphorylation of a particulate, possibly granular, membrane protein that may be of significance to the secretory process.

It is likely that PK C participates in the secretory response to cholinergic agents known to increase inositol phospholipid turnover and hence diacylglycerol formation in islets of Langerhans, since Malaisse & Sener (1985) showed that H7 inhibited the increment in insulin secretion caused by carbamoylcholine in the presence of glucose. Whether PK C is involved also in the secretory response to glucose itself is unresolved. In favour of this view, Dunlop & Larkins (1986) provided evidence that exposure of islets to glucose enhanced phosphorylation of a specific islet substrate for PK C, and Stutchfield *et al.* (1986) showed that polymyxin B inhibited equally the effects of glucose and TPA on insulin release. Moreover, a combination of phorbol ester and Ca^{2+} ionophore elicited biphasic insulin release essentially similarly to that induced by glucose (Zawalich *et al.*, 1983). On the other hand, the present study, in agreement with previous

Fig. 3. Effects of TPA and clomiphene on protein phosphorylation in intact islets

Islets were preincubated for 1 h in Hepes-buffered Krebs medium containing 100 μCi of [^{32}P]P_i (carrier free), 10 mM-glucose and BSA (2 mg/ml). Incubations were continued for a further 30 min in the absence or the presence of TPA (100 nM) (a), or in the presence of TPA, TPA and clomiphene (50 μM) or no additions (b). After incubation, islets were washed in Hepes-buffered Krebs medium. For the experiments in (a) and (b), the islets were then sonicated in 50 mM-sodium phosphate (pH 7)/50 mM-NaF/2 mM-EDTA/0.2 mM-EGTA/0.5 mM-PMSF/2 mM-benzamidine. For the experiment in (c), islets were homogenized in 0.3 M-sucrose/50 mM-sodium phosphate (pH 7)/1 mM-PMSF/2 mM-benzamidine, and fractionated by differential centrifugation to yield 600 g and 24000 g particulate fractions and a supernatant fraction. Samples were subjected to electrophoresis on SDS/16% acrylamide/0.5% AcrylAide gels. Dried gels were autoradiographed, and the Figure shows the densitometric traces of the autoradiograms (530 nm). The arrows indicate the position of the 36.7 kDa phosphopeptide.

findings (Malaisse & Sener, 1985), has shown that inhibition of PK C produced more marked effects on TPA-induced than on glucose-induced insulin release. Studies by Hii *et al.* (1987) have shown that PK C-

depleted islets retained a secretory response to glucose. These observations rather argue against a major role for PK C in the response to glucose itself.

Also unresolved are the cellular consequences of enhanced PK C-catalysed protein phosphorylation. Evidence both for (Dunne & Peterson, 1987) and against (Henquin *et al.*, 1987) effects on plasma-membrane ion fluxes has been presented. Synergism between TPA and the sulphonylurea gliclazide led to the suggestion that PK C activation increased Ca^{2+} mobilization (Malaisse *et al.*, 1983). However, other studies showing synergism between TPA and a Ca^{2+} ionophore led to the proposal that PK C allows sustained insulin secretion to occur at a lower intracellular Ca^{2+} concentration (Zawalich *et al.*, 1983). Direct evidence that activation of PK C by TPA does indeed alter the sensitivity of the β -cell to Ca^{2+} has come from two types of study. In islets permeabilized either by high-voltage electric discharge (Jones *et al.*, 1985) or by digitonin (Tamagawa *et al.*, 1985), TPA caused a dose-related shift in the Ca^{2+} -activation curve for insulin secretion to lower Ca^{2+} values; in intact islets we have similarly shown that TPA markedly decreases the dependence of glucose-stimulated insulin release on external Ca^{2+} (Hughes *et al.*, 1987).

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