A re-assessment of the role of protein kinase C in glucose-stimulated insulin secretion

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Isolated rat islets of Langerhans which had been pretreated with 200 nm-phorbol 12-myristate 13-acetate (PMA) for 20–24 h, a treatment reported in other cell types to deplete cells of protein kinase C activity, were found not to contain detectable Ca\(^{2+}\)/phospholipid-dependent protein kinase activity. These islets did not secrete insulin in response to a subsequent exposure to PMA (0.1 or 1 μM) during a 30 min incubation, although insulin secretion could be stimulated by 20 mM-glucose, a response which was enhanced by 20 μM-forskolin. PMA-pretreated islets that had been permeabilized by high-voltage discharge showed unimpaired secretory responses to an increase in Ca\(^{2+}\) concentration, cyclic AMP and forskolin. These results suggest that (i) pretreatment of islets with tumour-promoting phorbol esters may be a useful means of investigating the role of protein kinase C in stimulus-secretion coupling in the pancreatic β-cell and (ii) protein kinase C may not play an essential role in glucose-induced insulin secretion.

INTRODUCTION

Glucose, the most important physiological regulator of insulin secretion, causes many changes in the metabolism of pancreatic β-cells. Among these changes are rapid increases in the intracellular concentrations of Ca\(^{2+}\) (reviewed by Wollheim & Sharp, 1981) and cyclic AMP (reviewed by Sharp, 1979), which are thought to be important in mediating glucose-induced insulin secretion from the β-cells. More recently, attention has been focused on the possible role of the Ca\(^{2+}\)/phospholipid-dependent protein kinase, protein kinase C (PK C), in the regulation of insulin secretion. This enzyme has been identified in islet cells (Lord & Aschcroft, 1984), and intracellular concentrations of diacylglycerol, a physiological activator of PK C (reviewed by Nishizuka, 1984), are elevated during stimulation of insulin secretion from isolated islets by glucose (Peter-Riesch et al., 1986). Furthermore, 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; Hii et al., 1986) and from insulin-secreting cells (Hutton et al., 1984). However, the evidence for a role of PK C in glucose-induced insulin secretion is largely circumstantial. For example, Zawalich et al. (1983) reported that a combination of a Ca\(^{2+}\) ionophore and a phorbol ester produced a biphasic secretory response from isolated islets, which resembled that seen during glucose-induced insulin secretion, whereas Sturchfield et al. (1986) reported that the polyamine antibiotic polymyxin B, which inhibited PK C activity in islet extracts, inhibited glucose-stimulated insulin secretion.

Studies in other tissues suggest that total cellular PK C activity can be decreased by relatively long exposures (>18 h) to tumour-promoting phorbol ester (Rodriguez-Pena & Rozengurt, 1984; Gainer & Murray, 1985; Katakami et al., 1986; Klip & Ramlal, 1987). We have therefore used phorbol ester pretreatment to decrease PK C activity in isolated islets, and by this means to investigate the involvement of PK C in glucose-induced insulin secretion from intact islets and Ca\(^{2+}\)-induced secretion from electrically permeabilized islets.

EXPERIMENTAL

Materials

Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (4α-PDD), forskolin, collagenase type XI, diolein, phosphatidylserine (PS), lysine-rich histone type IIIS, phenylmethanesulphonyl fluoride, leupeptin, 2-mercaptoethanol and bovine insulin for iodination were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Rat insulin standards were purchased from Novo (Bagsvaerd, Denmark). Tissue-culture medium 199 (TCM 199), foetal-calf serum and Hanks buffered salts solution were from Gibco (Paisley, Scotland, U.K.). Antibiotics were from Glaxo (Greenford, Middx., U.K.). Filter discs (pore size 0.45 μm) were from Millipore Corp. [γ\(^{32}\)P]ATP (3000 Ci/mmol, carrier-free) was from New England Nuclear Corp. [Du Pont (U.K.), Stevenage, Herts., U.K.] and 125I (sodium salt) was from Amersham International (Amersham, Bucks., U.K.). All other reagents were of analytical grade from BDH (Poole, Dorset, U.K.).

Media

Hanks buffered salts solution, supplemented with streptomycin (100 μg/ml) and penicillin (100 units/ml), was used for islet isolation. TCM 199, supplemented with 10% (v/v) foetal-calf serum and the same concentrations of antibiotics as the Hanks solution, was...
used for islet culture. Static incubation experiments were performed by using either a bicarbonate-buffered medium (Gey & Gey, 1936) supplemented with 0.5 mg of bovine serum albumin/ml, 1 mM-CaCl₂ and 5.6 mM- or 20 mM-glucose, or a K⁺-glutamate-based Ca²⁺/EGTA buffer containing either 50 mM- or 10 mM-Ca²⁺ (Jones et al., 1985). The phorbol esters were dissolved in dimethyl sulphoxide (DMSO), and forskolin was dissolved in 95% (v/v) ethanol. Control islets received vehicle alone, the concentration of which did not exceed 0.1% (v/v).

Islet isolation and culture

Islets were isolated from fed Wistar rats (100–200 g) by a collagenase digestion technique (Howell & Taylor, 1968), washed (three times) by centrifugation (100 g, 30 s) and maintained in the presence of either 200 nm-PMA or 200 nm-4a-PDD at 37 °C in an atmosphere of humidified air/CO₂ (19:1). After 20–24 h the islets were harvested and washed (three times) in incubation buffer.

Insulin secretion

To study insulin secretion from intact islets, the islets were washed in Gey & Gey medium (5.6 mM-glucose) and incubated in groups of five in medium (0.5 ml) containing glucose (5.6 or 20 mM) and either 0.5 mM-PMA or 20 mM-forskolin. In experiments using permeabilized islets, the islets were washed in the K⁺-glutamate buffer (50 mM-Ca²⁺), permeabilized by five exposures to a high-voltage discharge (3.4 kV/cm), and incubated in groups of ten in buffer (1 ml) containing either 50 mM- or 10 mM-Ca²⁺, in the presence or absence of 0.5 mM-PMA, 20 mM-forskolin or 100 mM-cyclic AMP. After 30 min incubation at 37 °C, islets were sedimented by centrifugation (9000 g, 20 s), and supernatant samples were withdrawn and stored at −20 °C until assay. Insulin was measured by radioimmunoassay as described previously (Hii et al., 1986) by using guinea-pig anti-insulin serum, purified rat insulin as standard and 115I-labelled bovine insulin prepared by the Iodogen method (Salacinski et al., 1981).

Protein-phosphorylation assay

Groups of 1800–2200 islets were pelleted by centrifugation (9000 g, 20 s) and resuspended in 150 μl of ice-cold Tris/HCl buffer (20 mM, pH 7.5) containing 2 mM-EDTA, 5 mM-EGTA, 10 mM-2-mercaptoethanol, 0.2% Triton X-100, 0.01% leupeptin and 2 mM-phenylmethylsulphonyl fluoride. The islets were disrupted ultrasonically (3 × 10 s, 0 °C, setting 10 μm, MSE Soniprobe 150). The sonicated preparations were left for 1 h at 4 °C for enzyme extraction before centrifugation (27000 g, 30 min). The supernatant was removed and stored on ice before assay for kinase activity. The assay used was a modification of that described by Kitano et al. (1986). Briefly, the reaction mixture (50 μl) contained 1 μmol of Tris/HCl (pH 7.5), 0.25 μmol of magnesium acetate, 10 μg of histone type IIIIS, 0.5 nmol of [γ-³²P]ATP (0.9–1.2 Ci/mmol), 9.2 nmol of Ca²⁺, 1 μg of PS, and either 0.075 μg of dioloein or 0.025 nmol of PMA. When Ca²⁺ was omitted, 25 nmol of EGTA was added. The reaction was initiated by the addition of 10 μl of islet extract and was allowed to proceed for 3 min at 30 °C. The reaction was stopped by addition of 3 ml of 75% trichloroacetic acid, and acid-precipitable protein was retained on Millipore filter discs. The precipitates were washed with 10 ml of 25% trichloroacetic acid and ³²P radioactivity was measured by liquid-scintillation counting.

Statistics

Data are expressed as means ± s.e.m. Differences between treatments were assessed by Student's unpaired t test or one-way analysis of variance (ANOVA), as appropriate. Differences were considered significant when P < 0.05.

RESULTS

Protein phosphorylation

Sonicated detergent extracts of islets cultured in the presence of 200 nm-4a-PDD (control) contained Ca²⁺/phospholipid-dependent protein kinase activity. In the presence of 183 μM free Ca²⁺ and either PS/dioloein or PS/PMA, significantly more radioactivity was incorporated into histone type IIIIS than in the presence of Ca²⁺ alone (Fig. 1). In the absence of Ca²⁺ (1.5 mm-EGTA present), incorporation of ³²P was not stimulated by either PS/dioloein or PS/PMA. Pretreatment of the islets by 20–24 h exposure to 200 nm-PMA totally abolished the phospholipid-dependent phosphorylation of histone by extracts from PMA-pretreated islets, as shown in Fig. 1.
Table 1. Effects of phorbol ester pretreatment on insulin secretion from isolated islets

Islets were cultured in the presence of 0.02% DMSO, 200 nM-4α-PDD or 200 nM-PMA for 20–24 h as described in the Experimental section. After washing, the islets were incubated for 30 min under the conditions indicated above. Results are expressed as means ± S.E.M. for the numbers of observations indicated in parentheses. In the presence of 5.6 mM or 20 mM-glucose, PMA stimulated insulin secretion from islets pretreated with DMSO or 4α-PDD (ANOVA, \( P < 0.05 \)), but did not affect secretion from PMA-pretreated islets.

<table>
<thead>
<tr>
<th>Insulin secretion (ng/h per islet)</th>
<th>DMSO</th>
<th>4α-PDD-pretreated</th>
<th>PMA-pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6 mM-glucose</td>
<td>0.44 ± 0.07 (5)</td>
<td>0.48 ± 0.06 (18)</td>
<td>0.58 ± 0.07 (16)</td>
</tr>
<tr>
<td>+0.1 μM-PMA</td>
<td>0.88 ± 0.11 (5)</td>
<td>0.69 ± 0.09 (14)</td>
<td>0.55 ± 0.05 (14)</td>
</tr>
<tr>
<td>+1 μM-PMA</td>
<td>1.97 ± 0.18 (5)</td>
<td>1.49 ± 0.10 (23)</td>
<td>0.58 ± 0.06 (22)</td>
</tr>
<tr>
<td>20 mM-glucose</td>
<td>0.75 ± 0.11 (5)</td>
<td>0.74 ± 0.07 (23)</td>
<td>1.04 ± 0.09 (18)</td>
</tr>
<tr>
<td>+0.1 μM-PMA</td>
<td>1.97 ± 0.16 (5)</td>
<td>1.46 ± 0.04 (14)</td>
<td>1.24 ± 0.07 (14)</td>
</tr>
<tr>
<td>+1 μM-PMA</td>
<td>2.99 ± 0.22 (5)</td>
<td>2.28 ± 0.08 (23)</td>
<td>1.14 ± 0.09 (18)</td>
</tr>
</tbody>
</table>

Insulin secretion from intact islets

Table 1 shows the effects of phorbol ester pretreatment on the \( \beta \)-cell secretory responses to PMA and glucose tested during a subsequent 30 min incubation. In control islets pretreated with 4α-PDD, insulin secretion was stimulated by both PMA (0.1 and 1 μM) and glucose (20 mM). The response of these islets was not significantly different from that observed from islets cultured in the presence of DMSO (0.02%, v/v) alone (Table 1). Pretreatment with PMA totally abolished insulin secretion in response to a subsequent exposure to the phorbol ester. However, PMA pretreatment did not inhibit glucose-induced insulin secretion. On the contrary, PMA-pretreated islets responded to 20 mM-glucose with significantly higher insulin-secretory rates than those of control islets (\( P < 0.0005; \) Table 1).

Pretreatment of the islets with PMA for 20–24 h did not inhibit subsequent secretory responses to forskolin, an activator of adenylate cyclase. Fig. 2 shows the effects of forskolin on islets pretreated with either PMA or 4α-PDD. In 4α-PDD-pretreated islets, 20 μM-forskolin potentiated insulin secretion in response to 20 mM-glucose by approx. 3-fold, without significantly affecting insulin secretion by 5.6 mM-glucose. In PMA-pretreated islets, 20 μM-forskolin augmented insulin secretion by more than 3-fold in the presence of 5.6 mM-glucose and by more than 4-fold in the presence of 20 mM-glucose (Fig. 2). Note again that PMA-pretreated islets secreted more insulin than did 4α-PDD-pretreated islets in the presence of 20 mM-glucose (\( P < 0.05 \)).

Insulin secretion from permeabilized islets

To investigate whether phorbol ester pretreatment, and the subsequent decrease in PK C activity, affected the responsiveness of the islets to increases in intracellular Ca\( ^{2+} \), insulin secretion was measured from islets which had been permeabilized by exposure to a high-voltage discharge to allow direct manipulation of intracellular concentrations of Ca\( ^{2+} \). Raising the Ca\( ^{2+} \) concentration from 50 nM to 10 μM stimulated insulin secretion from permeabilized control islets. Pretreatment with PMA did not affect the secretory responses of permeabilized islets.

Table 2. Effects of PMA pretreatment on insulin secretion from electrically permeabilized islets

Islets were permeabilized and incubated as described in the Experimental section. Results are expressed as means ± S.E.M. for the numbers of observations indicated in parentheses. Significance of difference: \( * P < 0.001 \), \( ** P < 0.01 \), \( *** P < 0.05 \) versus 50 nM-Ca\( ^{2+} \).

<table>
<thead>
<tr>
<th>Insulin secretion (pg/h per islet)</th>
<th>4α-PDD-pretreated</th>
<th>PMA-pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nM-Ca( ^{2+} )</td>
<td>236 ± 31 (19)</td>
<td>269 ± 16 (22)</td>
</tr>
<tr>
<td>+0.5 μM PMA</td>
<td>547 ± 60 (19)*</td>
<td>305 ± 20 (23)</td>
</tr>
<tr>
<td>50 nM-Ca( ^{2+} )</td>
<td>258 ± 35 (12)</td>
<td>291 ± 19 (15)</td>
</tr>
<tr>
<td>+20 μM-forskolin</td>
<td>455 ± 56 (13)**</td>
<td>654 ± 54 (14)**</td>
</tr>
<tr>
<td>+100 μM-cyclic AMP</td>
<td>382 ± 59 (6)*****</td>
<td>565 ± 50 (6)*****</td>
</tr>
</tbody>
</table>

![Fig. 2. Effects of PMA pretreatment on the \( \beta \)-cell response to forskolin](image-url)

Islets were cultured and incubated as described in the Experimental section. Insulin secretion from 4α-PDD-pretreated (open bars) and PMA-pretreated (hatched bars) islets are expressed as means ± S.E.M. for six or seven observations. Significance of differences versus appropriate control: \( * P < 0.005 \) versus 5.6 mM-glucose; \( ** P < 0.001 \) versus 5.6 mM-glucose; \( t P < 0.01 \) versus 20 mM-glucose; \( t t P < 0.005 \) versus 20 mM-glucose.
to Ca\textsuperscript{2+}. Thus the rates of secretion in the presence of 50 nm-Ca\textsuperscript{2+} were 220 ± 28 pg/h per islet in α-PDD- and 262 ± 23 in PMA-pretreated islets (n = 18–22; N.S.); in the presence of 10 μM-Ca\textsuperscript{2+} the rates were 707 ± 78 in α-PDD- and 613 ± 43 in PMA-pretreated islets (n = 19–23; N.S.). Pretreatment with PMA totally abolished insulin secretion in response to 0.5 μM-PMA at a sub-stimulatory concentration of Ca\textsuperscript{2+}, as shown in Table 2. Both control and PMA-pretreated islets responded to 20 μM-forskolin and 100 μM-cyclic AMP after permeabilization. Note, however, that the secretory responses of PMA-pretreated islets to cyclic AMP and forskolin were significantly greater than those of controls (P < 0.05; Table 2).

**DISCUSSION**

Prolonged treatment of cells with tumour-promoting phorbol esters has been reported to cause a decrease or abolition of PK C activity, with the consequent loss of various cellular functions (reviewed by Murray et al., 1987). In the present study, similar effects have been shown to occur in isolated islets of Langerhans treated for 20–24 h with the phorbol ester PMA. Thus PMA-pretreated islets contained no detectable Ca\textsuperscript{2+}/PS-dependent kinase activity, and they did not respond to a subsequent exposure to PMA, in contrast with the marked stimulation by PMA of insulin secretion from islets which had been pretreated with an inactive phorbol ester.

Studies in other tissues suggest that prolonged PMA pretreatment may be a relatively specific means of down-regulating cellular PK C, since cellular functions which are lost after such treatment can be restored by micro-injection of purified PK C into the cells (Pasti et al., 1986). In the present study, pretreatment with PMA did not impair the secretory response of electrically permeabilized islets to increases in cytosolic Ca\textsuperscript{2+}, suggesting that the lack of response of PMA-pretreated islets to a subsequent PMA challenge was not due to a non-specific inhibition of the secretory process. These observations also imply that Ca\textsuperscript{2+}-induced insulin secretion from electrically permeabilized islets is not mediated by changes in intracellular PK C activity.

Similarly, PMA pretreatment did not inhibit the β-cell secretory response to increases in intracellular cyclic AMP, thought to be an important modulator of insulin secretion (reviewed by Sharp, 1979; Henquin, 1985). Thus forskolin stimulated insulin secretion from both control and PMA-pretreated intact islets, and both forskolin and cyclic AMP stimulated insulin secretion from permeabilized islets whether pretreated with PMA or the inactive analogue, 4α-PDD. Indeed, the present results suggest that PMA pretreatment, and the abolition of PK C activity, may enhance insulin secretion in response to elevations in intracellular cyclic AMP. The reasons for this increase in cyclic AMP-induced secretion are unclear. Activators of PK C have been reported to enhance basal and forskolin-stimulated adenylate cyclase activity in the 235-1 pituitary cell line (Summers & Cronin, 1986) and to block adrenaline-induced inhibition of adenylate cyclase in platelets (Watanabe et al., 1985). However, the enhancement of forskolin-induced insulin secretion from PMA-pretreated islets may not be a direct effect of PMA pretreatment on islet adenylate cyclase, since the secretory response of permeabilized islets to cyclic AMP was also enhanced, perhaps suggesting a sensitization of the secretory process to increases in intracellular cyclic AMP.

Nutrient secretagogues have been reported to stimulate the hydrolysis of phosphoinositides by phospholipase C (Best & Malaise, 1983, 1984; Montague et al., 1985). The ability of tumour-promoting phorbol esters to stimulate insulin secretion has led to the suggestion that activation of PK C by diacylglycerol, transiently released during phosphoinositide breakdown, may play an important role in regulating nutrient (e.g. glucose)-induced insulin secretion (Zawalich et al., 1983; Hubinont et al., 1984). In support of such a role were the observations that, in isolated islets, PMA caused a delayed stimulation of insulin secretion similar to the second phase of glucose-induced insulin secretion (Zawalich et al., 1983) and that glucose-stimulated insulin release could be inhibited by polyamines (Thams et al., 1986; Stutchfield et al., 1986), which have been shown to inhibit PK C in a number of cell types (Wise et al., 1982; Qi et al., 1983; Thams et al., 1986). However, the results of the present study argue against a major role for PMA/diolein-stimulatable PK C as a regulator of glucose-induced insulin secretion, since PMA-pretreated islets responded to glucose even though the pretreatment abolished PMA/diolein-stimulated PK C activity and inhibited insulin secretion in response to a subsequent exposure to the phorbol ester. Indeed, the secretory response to glucose was consistently greater in PMA-pretreated islets than in control islets treated with the inactive 4α-PDD, perhaps reflecting a greater sensitivity of PMA-pretreated islets to changes in other intracellular mediators such as cyclic AMP, the concentration of which is increased during glucose stimulation (reviewed by Sharp, 1979). The stimulation of insulin secretion from PMA-pretreated islets by forskolin in the presence of a non-stimulatory glucose concentration is consistent with this suggestion, since forskolin normally only stimulates insulin secretion in the presence of stimulatory concentrations of glucose (reviewed by Henquin, 1985), as was observed for the 4α-PDD-treated islets in the present study.

The discrepancies between the results of the present study and previous studies with polyamine inhibitors of PK C (Thams et al., 1986; Stutchfield et al., 1986) may lie in the lack of specificity of polyamines as PK C inhibitors. There are several reports that, in addition to inhibiting PK C, polyamines inhibit Ca\textsuperscript{2+}/calmodulin-dependent kinases (Mazzei et al., 1982; Qi et al., 1983), which have been implicated in the control of glucose-induced insulin secretion (reviewed by Harrison et al., 1984). The observation that Ca\textsuperscript{2+}-induced insulin secretion from electrically permeabilized islets was inhibited by polymyxin B (Stutchfield et al., 1986) is consistent with these reports.

In summary, exposing isolated islets of Langerhans to low concentrations of a tumour-promoting phorbol ester for 20–24 h resulted in down-regulation of cellular PK C activity. Treated islets did not secrete insulin in response to a subsequent exposure to the phorbol ester, although the responses to glucose, forskolin, cyclic AMP or Ca\textsuperscript{2+} were not impaired. These results suggest that the activation of PK C may not play an essential role in stimulus–secretion coupling in glucose-stimulated β-cells, although it may be involved in stimulation by secretagogues other than glucose.
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REFERENCES


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