Phorbol ester stimulation of insulin release and secretory-granule protein phosphorylation in a transplantable rat insulinoma

John C. HUTTON,* Mina PESHAVARIA and Keith W. BROCKLEHURST†

Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.

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The effects of tumour-promoting phorbol esters on protein-phosphorylation reactions and secretion in rat insulinoma tissue were investigated with the objective of assessing the possible role of Ca^{2+} - and phospholipid-dependent protein kinases (protein kinase C) in insulin release. 4β -Phorbol 12-myristate 13-acetate (TPA) was a potent secretagogue at concentrations above $0.1\ \mu\text{M}$. TPA-induced release was inhibited by adrenaline or omission of Ca^{2+} from the extracellular medium and was augmented by theophylline. These findings suggested that TPA activated an exocytotic process. TPA enhanced the Ca^{2+} - and phospholipid-dependent phosphorylation of histone III-S by a soluble protein fraction of the tissue. Endogenous phosphorylation reactions involving soluble and secretory-granule membrane proteins were also stimulated by TPA in tissue homogenates and reconstituted subcellular fractions. Histone phosphorylation and the granule-protein phosphorylation reactions showed similar concentration-dependencies for activation by both Ca^{2+} and TPA, thus indicating that the same enzyme was involved. It is concluded that the phosphorylation of cytosolic and membrane protein substrates by protein kinase C may be important in the stimulus-secretion coupling mechanism of insulin release.

The release of insulin from the pancreatic B-cell appears to be controlled by three convergent and interdependent regulatory pathways involving changes in the cytosolic Ca^{2+} , intracellular cyclic nucleotide concentrations and in the composition of membrane-associated lipids (Wollheim & Sharp, 1981; Best & Malaisse, 1983). The transduction of these signals in a variety of cells occurs by changes in the phosphorylation state of specific proteins, and it is conceivable that phosphorylation reactions involving contractile elements, the secretory granules or plasma membranes provide the molecular basis for the regulation of exocytosis in insulin secreting tissue.

Glucose is reported to stimulate the phosphorylation of several proteins in intact islets of Langerhans (Suzuki *et al.*, 1981), and islet homogenates exhibit cyclic-AMP- and Ca^{2+} -dependent protein phosphorylation of endogenous substrates (Harrison & Ashcroft, 1982). Using

Abbreviation used: TPA, 4β -phorbol 12-myristate 13-acetate.

* To whom correspondence should be addressed.

† Present address: National Institutes of Health, Bethesda, MD, U.S.A.

purified subcellular fractions derived from a transplantable rat insulinoma, we have identified three Ca^{2+} -dependent phosphorylation reactions involving the secretory-granule membrane (Brocklehurst & Hutton, 1983). These occur after a Ca^{2+} -dependent interaction of cytosolic and secretory-granule membrane proteins, and are inhibited by trifluoperazine, but are not affected by manipulation of the calmodulin concentration in the incubation. These findings indicated the possible involvement of a Ca^{2+} - and phospholipid-dependent enzyme (protein kinase C) in these reactions, similar to that described in other tissues (Minakuchi *et al.*, 1981).

Protein kinase C may be activated by diacylglycerols (Kishimoto *et al.*, 1980), an effect which appears to be mimicked by tumour-promoting phorbol esters (Castagna *et al.*, 1982). The latter compounds stimulate secretion in various tissues, including the pancreatic B-cell (Virji *et al.*, 1978; Malaisse *et al.*, 1980). In the present context such components provide an experimental approach to assessing the involvement of protein kinase C activity in insulin-secretory-granule phosphorylation and its relevance to the secretory mechanism.

Materials and methods

Phorbol and its esters were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K., and were introduced into media as 500-fold concentrated solutions in dimethyl sulphoxide; this solvent did not affect any of the parameters determined. [γ - 32 P]ATP was prepared from carrier-free [32 P]P_i (Amersham International, Amersham, Bucks., U.K.) by the method of Glynn & Chappell (1964). Insulinoma tissue derived from an X-ray-induced pancreatic tumour (Chick *et al.*, 1977) was propagated in NEDH-strain rats and harvested as previously described (Hutton *et al.*, 1981).

Insulin-secretion experiments

Suspensions of cells were prepared by digesting tumour pieces (about 2g wet wt.) for 30 min at 37°C with intermittent shaking in 3 ml of Krebs–Henseleit bicarbonate buffer (Dawson *et al.*, 1978) containing 5.6 mM-glucose, 5 mg of bovine plasma albumin/ml, 2.5 mg of collagenase (Boehringer Corp., Lewes, Sussex, U.K.)/ml and 0.1 mg of deoxyribonuclease 1 (Sigma)/ml. The cells were recovered by centrifugation at 300g for 3 min, washed twice with medium without collagenase, filtered through 500 μ m- and 50 μ m-mesh nylon sieves (LKB, Stockholm, Sweden) and washed a further three times as above. The viability of the preparation, assessed by Trypan Blue exclusion, was greater than 85%. Cells were preincubated for 1 h at 37°C under an O₂/CO₂ (19:1) atmosphere in Krebs–Henseleit buffer containing 2.8 mM-glucose, 0.1 mg of bovine plasma albumin/ml and 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4. They were again washed and then incubated at 37°C in 1 ml at approx. 1 mg of cellular protein/ml in the same buffer containing the various additions indicated. The cells and medium were separated after 15 min by centrifugation at 800g for 5 min and assayed for insulin (Hutton *et al.*, 1982) and lactate dehydrogenase activity. The latter enzyme was determined in an indirect fluorimetric assay based on the method of Niellands (1955).

Subcellular fractionation

A homogenate of insulinoma pieces was prepared in a solution containing 0.27 M-sucrose, 1 mM-EGTA and 10 mM-Mes (4-morpholine-ethanesulphonic acid) adjusted to pH 6.5 with Tris. Insulin granules were prepared from this homogenate by density-gradient centrifugation in Percoll-containing media by the method of Hutton *et al.* (1982), and were stored at -70°C in the same medium but without EGTA. A soluble protein fraction was prepared by centrifugation of the

homogenate at 230 000g for 30 min at 4°C in a Beckman SW 50.1 rotor.

Phosphorylation assays

Subcellular fractions (50 μ g of soluble protein and/or 100 μ g of granule protein) were incubated for 30–45 s in 50 μ l of a solution containing 50 mM-Mes, 1 mM-MgCl₂, 0.1 mM-dithiothreitol, 5 mM-EGTA and 0.1 mM-[γ - 32 P]ATP (500–2000 Ci/mol) adjusted to pH 6.9 with Tris. CaCl₂ was included to give free Ca²⁺ concentrations in the range 0.2–150 μ M, the actual concentrations being computed as previously described (Hutton *et al.*, 1982) and confirmed by Ca²⁺-electrode determinations.

Protein kinase C activity was determined under identical conditions by further addition of calf thymus histone type III-S (0.3 mg/ml; Sigma) and bovine brain phosphatidylserine (Sigma; 80 μ g/ml) to the reaction. The latter was introduced as a dispersion produced by sonication of the compound in 10 mM-Mes, pH 6.5.

Reactions were terminated by the addition of 30 μ l of a solution containing 0.24 M-Tris/HCl, pH 6.8, 0.86 M-sucrose, 3.8% (w/v) sodium dodecyl sulphate and 0.02% Bromophenol Blue, and heating at 100°C for 5 min. Samples were then electrophoresed on polyacrylamide slab gels prepared from 19% (w/v) acrylamide and 0.07% bisacrylamide in the buffer system of Laemmli (1970). Gels were stained with Kenacid Blue, dried and autoradiographed on preflashed film. Quantification of endogenous protein phosphorylations was performed by densitometric scanning of the autoradiographs (Chromoscan 3; Joyce-Loebl and Co., Gateshead, Tyne and Wear, U.K.). In the histone-phosphorylation assay, pieces of the gel corresponding to the histone bands were excised and suspended in 5 ml of water, and their Čerenkov radioactivity was determined with a liquid-scintillation counter. The time courses of Ca²⁺-dependent phosphorylation of both histone and the specific granule proteins mentioned in the Results section were linear under the conditions used.

Protein was determined in samples after precipitation with 5% (w/v) trichloroacetic acid by the method of Lowry *et al.* (1951), with bovine plasma albumin as standard. All representative experiments illustrated were repeated on three or more occasions.

Results

Insulin release

Insulin release by insulinoma-cell suspensions was stimulated by TPA at concentrations of 0.1 μ M and above (Fig. 1). The maximal insulinotropic response to the compound was similar in magnitude to that induced by the combination of leucine

and theophylline, the most potent secretagogue combination previously reported by us for these cells (Sopwith *et al.*, 1981). Release in the combined presence of TPA and theophylline was greater than with either secretagogue alone, although the effects were less than additive (Table 1). The response to TPA was inhibited by omission of Ca^{2+} from the medium or by the addition of adrenaline, a finding which suggested that TPA was activating an exocytotic process rather than acting as a lytic agent. This was further supported by the observation that the release into the medium of the cytosolic enzyme lactate dehydrogenase ($2.8 \pm 0.2\%/15\text{min}$ under basal conditions; 4

experiments) was not altered by TPA. The related compounds 4α -phorbol and 4α -phorbol 12,13-didecanoate, which have a lesser tumour-promoting activity than TPA (Boutwell, 1974), failed to affect insulin release when used at $0.1\text{ }\mu\text{M}$ concentrations. The insulinotropic response of these insulinoma cells to TPA was thus comparable with that reported for islets of Langerhans (Virji *et al.*, 1978; Malaisse *et al.*, 1980).

Protein kinase C assays

The phosphorylation of exogenous histone by an insulinoma soluble protein fraction determined in the presence of low Ca^{2+} concentrations ($<1\text{ }\mu\text{M}$) was not affected by the addition of phosphatidylserine alone, but was more than doubled in the combined presence of phosphatidylserine and TPA (Fig. 2a). TPA alone had an intermediate effect. When the Ca^{2+} concentration exceeded $10\text{ }\mu\text{M}$, phosphatidylserine alone increased histone phosphorylation 8-fold, an effect which was further enhanced by TPA addition. TPA alone increased phosphorylation approx. 3-fold. Under each of these conditions, the Ca^{2+} -dependent response in phosphorylation was half-maximal at approx. $10\text{--}20\text{ }\mu\text{M}$. The stimulatory effects of TPA observed in these experiments at $0.1\text{ }\mu\text{M-Ca}^{2+}$ were still evident in the presence of 1mM-Ca^{2+} , suggesting that at least part of the effects of TPA did not depend on this cation (J. C. Hutton, M. Peshavaria & K. W. Brocklehurst, unpublished work).

Stimulatory effects of TPA addition on histone phosphorylation were observed at concentrations above 10 nM and were maximal at concentrations of $0.1\text{--}1\text{ }\mu\text{M}$ (Fig. 2b). Similar concentration-dependencies were observed in the presence or absence of phosphatidylserine at low Ca^{2+} concentrations and in the absence of phosphatidylserine at higher Ca^{2+} concentrations.

Histone phosphorylation by the soluble protein

Fig. 1. Concentration-dependency of TPA-induced insulin secretion in rat insulinoma-cell suspensions
Duplicate incubations were performed for 15 min at 37°C in Krebs-Henseleit buffer containing 2.8 mM -glucose. Each value is the mean $\pm$ S.E.M. of results obtained in three experiments. Secretion in the absence of TPA was $2.45 \pm 0.43\%$ of the cellular insulin content (1.4 ± 0.1 units/mg of protein).

Table 1. Insulin release from tumour cells induced by TPA

Incubations were performed for 15 min at 37°C in Krebs-Henseleit medium containing 2.8 mM -glucose and the indicated concentrations of test substances. Ca^{2+} omission was accompanied by the addition of 1 mM-NaEGTA . The tabulated values are the means $\pm$ S.E.M. of results obtained in duplicate incubations; the numbers of separate experiments performed are indicated in parentheses. The statistical significances of differences estimated by Student's *t* test for unpaired data refer in each case to the incubation at the corresponding TPA concentration: * $P < 0.001$; ** $P < 0.01$.

Modification		Insulin release (% of cellular content)	
		Control	+ $0.1\text{ }\mu\text{M-TPA}$
None	(10)	2.77 ± 0.25	4.16 ± 0.16
Theophylline (10 mM)	(8)	$4.41 \pm 0.19^*$	$5.06 \pm 0.27^{**}$
Ca^{2+} omission	(4)	2.32 ± 0.09	$2.42 \pm 0.25^*$
Adrenaline ($5\text{ }\mu\text{M}$)	(4)	2.11 ± 0.12	$2.83 \pm 0.08^*$

Fig. 2. Histone III-S phosphorylation by an insulinoma soluble-protein fraction

(a) Incubations were performed for 45 s at 30°C at the indicated Ca^{2+} concentrations either without further additions (○), or with the addition of 0.1 μM -TPA (●), phosphatidylserine (80 $\mu\text{g}/\text{ml}$) (△), or TPA plus phosphatidylserine (▲). Results were expressed as percentages of the radioactivity incorporated in the incubation performed in the presence of TPA, phosphatidylserine and 0.1 mM- Ca^{2+} (typically 8000–10000 c.p.m.). Each value represents the mean of three separate experiments. (b) Incubations were performed for 45 s at 30°C at the indicated TPA concentrations with the addition of 15 μM - Ca^{2+} (●), phosphatidylserine (80 $\mu\text{g}/\text{ml}$) in the absence of Ca^{2+} (△) or without either effector (○). Results were expressed as percentages of the radioactivity incorporated in the incubation performed without TPA, phosphatidylserine or Ca^{2+} (▲). Each value is the mean of results obtained in three experiments.

fraction in the presence of 15 μM - Ca^{2+} increased progressively with phosphatidylserine addition: a maximal effect was observed at 100 $\mu\text{g}/\text{ml}$ and a half-maximal response occurred at 12 $\mu\text{g}/\text{ml}$. It should be noted, however, that phosphatidylserine in these incubations was in an emulsified form.

Phosphorylation of endogenous proteins

The effects of the addition of Ca^{2+} , phosphatidylserine and TPA on the phosphorylation of endogenous proteins in a soluble protein fraction, in secretory granules and in a reconstituted soluble-protein/granule fraction are illustrated in Fig. 3. These experiments were performed at a sub-maximally effective Ca^{2+} concentration (5 μM) so that the effects of TPA and phosphatidylserine were more readily detectable.

In a soluble protein fraction, addition of Ca^{2+} stimulated the phosphorylation of a M_r -57000 protein, as described previously (Brocklehurst & Hutton, 1983). Addition of TPA to the soluble protein fraction either with or without Ca^{2+} did not alter the respective phosphorylation patterns. Addition of phosphatidylserine, however, resulted in the Ca^{2+} -dependent phosphorylation of a series of new proteins, of M_r 17000, 18000, 25000, 26000 and 36000. TPA combined with phosphatidylserine promoted further Ca^{2+} -dependent phosphorylation of these proteins. In the absence of Ca^{2+} , TPA combined with phosphatidylserine also increased the background extent of phosphorylation compared with TPA or phosphatidylserine alone.

Insulin-secretory granules alone did not exhibit Ca^{2+} -dependent phosphorylation reactions in either the presence or the absence of TPA. When secretory granules were combined with the soluble protein fraction, however, three Ca^{2+} -dependent phosphorylation reactions were evident which were not observed in either fraction alone. These reactions have been previously characterized with regard to their subcellular localization (Brocklehurst & Hutton, 1983). The phosphorylation of these peptides was further enhanced by the addition of TPA in the presence of Ca^{2+} . Phosphatidylserine addition to the incubation resulted in a decrease in the Ca^{2+} -dependent phosphorylation of two of these proteins (M_r 29000 and 100000) while increasing that of the other (M_r 10000).

The Ca^{2+} -concentration-dependencies of the phosphorylation reactions of the M_r -10000, -29000 and -100000 proteins catalysed by the soluble-protein/granule mixture (Fig. 4, upper panel) were similar in that maximal phosphorylation was observed at Ca^{2+} concentrations in the 50–100 μM range and that the half-maximally effective concentrations were of the order of 10–20 μM . In each case, TPA approximately halved the Ca^{2+} concentration required for half-maximal phosphorylation and increased the phosphorylation in the absence of Ca^{2+} . With the M_r -29000 and -100000 proteins, but not the M_r -10000 protein, TPA also increased the maximal extent of phosphorylation observed in the presence of Ca^{2+} .

Fig. 3. Endogenous protein phosphorylation in insulinoma soluble-protein fraction, in secretory granules and in soluble-protein/granule mixtures

Incubations were performed for 30 s at 30°C in either the absence or the presence of Ca²⁺ (5 μM) and with or without the further addition of 0.1 μM-TPA or phosphatidylserine (80 μg/ml) as indicated.

The TPA-concentration-dependencies of the phosphorylation reactions of the *M_r*-10 000, -29 000 and -100 000 proteins in the granule/soluble-protein mixtures (Fig. 4, lower panel) were similar to one another and comparable with that of the phosphorylation of histone catalysed by the soluble protein fraction alone (Fig. 2*b*). With either the endogenous proteins or histone as substrates, the half-maximally effective TPA concentration was approx. 50 nM. 4α-Phorbol and 4α-phorbol 12,13-didecanoate at concentrations of 0.1 μM did not affect endogenous phosphorylation reaction in either the presence or the absence of Ca²⁺ catalysed by either the soluble-protein fraction or the soluble-protein/granule mixture.

Discussion

Ca²⁺- and phospholipid-dependent protein kinase activity (protein kinase C), described initially in brain (Inoue *et al.*, 1977), has a widespread

distribution with respect to both tissue and phyla (Kuo *et al.*, 1980; Minakuchi *et al.*, 1981). Isoenzymic forms of the activity may exist in different tissues (Wise *et al.*, 1982; Kikkawa *et al.*, 1982; Schatzman *et al.*, 1983). The enzyme is present in rat islets (Tanigawa *et al.*, 1982) and rat insulinoma tissue (Brocklehurst & Hutton, 1984). It has been partially purified from a hamster insulinoma B-cell line and found to possess catalytic and physical properties similar to that of the heart isoenzyme (Lord & Ashcroft, 1984). Protein kinase C catalyses the phosphorylation of a variety of endogenous proteins of a wide *M_r* range located in both soluble and particulate fractions of different tissues. Myosin light chains (Naka *et al.*, 1983) and troponins I and T (Katoh *et al.*, 1982) have been identified as possible physiological substrates. Functional significance of protein kinase C-mediated phosphorylation in exocytosis has been documented in platelets (Nishizuka, 1983).

Fig. 4. Phosphorylation of endogenous proteins of M_r 10000 (a), 29000 (b) and 100000 (c) catalysed by soluble-protein/granule-fraction mixtures

Upper panel: incubations were performed for 30 s at 30°C at the indicated Ca^{2+} concentrations either with (●) or without (○) the addition of 0.1 μM -TPA. Lower panel: incubations were performed for 30 s at 30°C at the indicated TPA concentrations in either the absence (○) or the presence of 5 μM - Ca^{2+} (●). Results were expressed as percentages of the maximal absorbance observed for each protein on suitably exposed autoradiographs. Each value is the mean of results obtained in two experiments.

The rat insulinoma enzymic activity characterized by us showed certain features in common with the rat brain soluble enzyme (Castagna *et al.*, 1982). For example, the concentration of Ca^{2+} required for half-maximal activity was of the same order (20 μM and 70 μM respectively), and nanomolar concentrations of TPA activated the insulinoma and brain enzymes. However, TPA did not substantially lower the Ca^{2+} concentration required for half-maximal activation of the insulinoma enzyme, as is the case for the brain protein.

An alternative pathway of activation of protein kinase C is through Ca^{2+} -dependent limited proteolysis of the enzyme. This process is also enhanced by the presence of phospholipid and diacylglycerols acting on the protein substrate. However, the product of the reactions (protein kinase M) is no longer sensitive to Ca^{2+} , phospholipid, diacylglycerol and phorbol esters (Castagna *et al.*, 1982; Kishimoto *et al.*, 1983). Such a process would not explain the anomalous behaviour of the

insulinoma enzyme activity with respect to the limited effect of TPA on the K_s for Ca^{2+} or the finding that a component of the TPA-activated phosphorylation was independent of Ca^{2+} . Experiments conducted with membrane preparations from A431 (Moon *et al.*, 1984) and U-937 (Grunberger *et al.*, 1984) cells demonstrate that TPA may activate the Ca^{2+} -independent phosphorylation of tyrosine residues of several exogenous and endogenous proteins. In the former case the involvement of protein kinase C appears to be excluded. It is therefore likely that the insulinoma soluble-protein fraction studied contains, apart from an activity resembling brain protein kinase C, other related and unrelated TPA-sensitive enzymes. Further purification of these enzymes would therefore seem warranted.

Several cytosolic protein substrates for protein kinase C activity were identified in the insulinoma on the basis of the effects of Ca^{2+} , phosphatidylserine and TPA additions on the incorporation of [γ - ^{32}P]ATP radioactivity into endogenous con-

stituents of a soluble protein fraction. One such reaction, involving a peptide of M_r 26000, was previously described as a Ca^{2+} -dependent phosphorylation reaction in a crude homogenate of insulinoma (Brocklehurst & Hutton, 1983). In that instance, presumably, the endogenous phospholipids were capable of activating the kinase.

Other protein kinase C substrates were identified in association with the insulin-secretory granule, the proteins (M_r 10000, 29000 and 100000) being those previously identified as undergoing Ca^{2+} -dependent but calmodulin-independent phosphorylation in granule/soluble-protein mixtures (Brocklehurst & Hutton, 1983). The involvement of protein kinase C in the phosphorylation reaction of the M_r -29000 protein has been previously documented (Brocklehurst & Hutton, 1984). The phosphorylation of these substrates was stimulated by Ca^{2+} and TPA at concentrations similar to those affecting histone phosphorylation, a finding which suggested that the same enzyme was involved. The enzyme activity responsible for the phosphorylation of the granule-associated substrates was again dissimilar to brain protein kinase C in not showing a marked decrease in K_m for Ca^{2+} in the presence of TPA. Phosphatidylserine affected the granule-associated phosphorylations in a complex manner, inhibiting that of the M_r -29000 and -100000 proteins while stimulating the phosphorylation of the M_r -10000 protein. Exogenous phosphatidylserine might be expected to compete with the endogenous granule phospholipid as a binding site for protein kinase C, and it is conceivable that either stimulatory or inhibitory effects on the phosphorylation of membrane proteins would result from phosphatidylserine addition, depending on the accessibility of the substrate to the activated enzyme/phospholipid complex. It is noteworthy in this regard that the M_r -29000 substrate has been identified as an intrinsic-granule membrane protein, whereas the M_r -10000 substrate, although bound to the granule in the unphosphorylated state, dissociates from it on phosphorylation (Brocklehurst & Hutton, 1983).

The biological effects of the phorbol esters in various different cells may be the consequence of structural similarities between these compounds and tissue diacylglycerols which, like TPA, are also potent activators of protein kinase C (Kishimoto *et al.*, 1980). The content of diacylglycerol in membranes of the pancreatic B-cell, as in other tissues, is thought to be a function of the breakdown and resynthesis of (poly)phosphoinositides (see Nishizuka, 1984). Changes in intracellular Ca^{2+} concentration and phosphoinositide turnover occur during insulin secretion (see Wollheim & Sharp, 1981; Best & Malaisse, 1983). The observations that

TPA is a potent secretagogue in islets (Virji *et al.*, 1978; Malaisse *et al.*, 1980) and insulinoma cells, and that these tissues contain protein kinase C and several endogenous substrates for protein kinase C, suggests a possible role for this enzyme in the stimulus-secretion coupling mechanism.

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