Activation of protein kinase C by a tumor-promoting phorbol ester in pancreatic islets

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Rat pancreatic islet homogenates display protein kinase C activity. This phospholipid-dependent and calcium-sensitive enzyme is activated by diacylglycerol or the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In the presence of TPA, the K_a for Ca^{2+} is close to 5 μM. TPA does not affect phosphoinositide turnover but stimulates [^{32}P]- and [^{3}H]choline-labelling of phosphatidylcholine in intact islets. Exogenous phospholipase C stimulates insulin release, in a sustained and glucose-independent fashion. The secretory response to phospholipase C persists in media deprived of $CaCl_2$. It is proposed that protein kinase C participates in the coupling of stimulus recognition to insulin release evoked by TPA, phospholipase C and, possibly, those secretatogues causing phosphoinositide breakdown in pancreatic islets.

Pancreatic islet *Protein kinase C* *Phospholipase C* *Tumor-promoting phorbol ester*

1. INTRODUCTION

In the pancreatic B-cell, protein kinases are thought to play an important role in the coupling of stimulus recognition to insulin release. Three types of protein kinases have already been identified in pancreatic islet cells, namely the cyclic AMP-responsive protein kinase A [1–3], a Ca-calmodulin-sensitive kinase [4–8] and the phospholipid-dependent Ca^{2+}-responsive protein kinase C which is activated by unsaturated diacylglycerol [9]. This work deals with both the activation of the latter enzyme by the tumor-promoting agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the possible participation of protein kinase C in stimulus-secretion coupling.

2. MATERIALS AND METHODS

All experiments were performed with isolated islets removed from the pancreas of fed albino rats [10].

2.1. *Assay of protein kinase*

For the assay of protein kinase C, batches of 500–1000 islets were sonicated (3 times 5 s) in a Tris–HCl buffer (50 mM; pH 7.5) containing EGTA (0.2 mM), $MgCl_2$ (10 mM), 2-mercaptoethanol (50 mM) and Triton X-100 (0.1%, v/v), in a ratio of about 500 islets per ml. Aliquots (50 μl) of this crude homogenate were added to 150 μl of a reaction mixture to yield the following final concentrations: Tris–HCl (50 mM; pH 7.5), EGTA (0.2 mM), $MgCl_2$ (10 mM), 2-mercaptoethanol (12.5 mM), Triton X-100 (0.025%, v/v), lysine-rich histone from calf thymus (0.2 mg/ml; type III-S, Sigma, St. Louis, MO), ATP (0.1 mM; sodium salt) mixed with a tracer amount of [γ-^{32}P]ATP (6 μCi/ml; Amersham International, Buckinghamshire) and, as required, L-α-phosphatidyl-L-serine (25 μg/ml) emulsified by sonication in a Tris–HCl buffer (see above) containing bovine albumin (0.1 mg/ml), 1,3-diolein (1 μg/ml or 1.6 μM; solubilized in DMSO at an initial concentration of 10 mg/ml; Sigma), TPA (20 nM; PL Biochemicals, Milwaukee, WI) and/or the com-

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bination of cyclic AMP (10 μM) and theophylline (2.5 mM). The Ca^{2+} concentration of the reaction mixture was adjusted by addition of $CaCl_2$ (0.2–0.7 mM). After 2 min preincubation, the reaction was initiated by the addition of ATP and, after 5 min incubation at 30°C, terminated by the rapid and successive addition of a solution (200 μl) of bovine albumin (10 mg/ml) in Tris–HCl buffer (see above) and 1.0 ml trichloroacetic acid (10%, w/v). After centrifugation, the precipitate was redissolved in 1.0 ml NaOH (0.5 M) and TCA (1.0 ml) was again added. After 5 such successive washes, the final precipitate was redissolved in NaOH and examined for its radioactive content by liquid scintillation. Blank values were measured in the absence of islet homogenate.

The assay of protein kinase A was performed in a similar manner. The islets were sonicated in the same Tris–HCl buffer (about 750 islets/ml) containing EGTA (0.2 mM) and $MgCl_2$ (10 mM) but neither mercaptoethanol nor Triton X-100. The type of histones (type IIA; Sigma) and their final concentration (2.5 mg/ml) were also different from those used in the assay of protein kinase C.

In the assay of either protein kinase C or A, the incorporation of ^{32}P into trichloroacetic acid-precipitable material was related to the amount of tissue ($r = 0.906$, $n = 7$). The reaction velocity was not linear with time, a situation possibly attributable to interference of protein phosphatase. The time of incubation was restricted, therefore, to either 5 (protein kinase C) or 10 min (protein kinase A). All determinations were made in triplicate, and the results expressed relative to the mean basal value found within the same experiment.

2.2. *Phospholipid metabolism*

Islet phospholipids were labelled by preincubation with either $^{32}P_i$ (carrier-free), *myo*-[2-^{3}H]inositol (16.6 Ci/mmol), [1(3)-^{3}H]glycerol (3 Ci/mmol) or [*methyl*-^{3}H]choline chloride (60 Ci/mmol). The radioactivity of the preincubation medium ranged from 5–100 μCi/ml. The methods used for separation of islet phospholipids and their metabolites are described in detail in [11,12].

2.3. *Insulin release*

Insulin release from intact islets was measured as in [13]. Two preparations of phospholipase C extracted from *Clostridium perfringens* (Sigma) and *Bacillus cereus* (Boehringer, Mannheim), respectively, were used.

2.4. *Presentation of results*

All results are expressed as the mean (± SE) together with the number of individual determinations (n). The statistical significance of differences between mean values was assessed by the use of Student's t-test.

3. RESULTS

3.1. *Protein kinase activity in islet homogenates*

In a first series of experiments, lysine-rich histones were used as the substrate for protein kinase C. The islet homogenates catalyzed the incorporation of ^{32}P from [γ-^{32}P]ATP into such histones. The reaction velocity failed to be significantly affected by the presence of Ca^{2+} and/or the combination of cyclic AMP and theophylline (table 1, lines 1–4). In the absence of Ca^{2+}, diolein or TPA also failed to affect significantly the incorporation of ^{32}P in the histone (table 1, lines 7,9). In the absence of Ca^{2+}, the presence of phosphatidylserine tended to slightly increase the incorporation of ^{32}P into trichloroacetic acid-precipitable material (table 1, lines 5,11,13,16), but failed to confer any Ca^{2+}-sensitivity to the assay system (table 1, lines 5,6). In the absence of exogenous phospholipid but presence of diolein, Ca^{2+} tended to decrease ^{32}P incorporation into the trichloroacetic acid-precipitable material (table 1, lines 7,8). Inversely, TPA in the absence of exogenous phospholipid apparently conferred to the system a limited but significant ($p < 0.001$) and positive reactivity towards Ca^{2+} (table 1, lines 9,10). In the presence of phosphatidylserine, diolein and, even more so, TPA caused obvious stimulation of enzyme activity provided that Ca^{2+} was also present in the incubation medium (table 1, lines 11–14). Diolein failed to further enhance the response to TPA in media containing both phosphatidylserine and Ca^{2+} (table 1, lines 14,15). Likewise, cyclic AMP and theophylline failed to affect significantly the reaction velocity recorded in the combined presence of TPA, phosphatidylserine and Ca^{2+} (table 1, lines 14,17). In the presence of

Table 1

Presence of protein kinase C activity in islet homogenates

Line	Phosphatidyl-serine (25 μg/ml)	Diolein (1.6 μM)	TPA (20 nM)	Cyclic AMP[a] (10 μM)	Ca^{2+} (0.5 mM)	Reaction velocity[b] (%)
1	–	–	–	–	–	100.0 ± 4.8 (10)
2	–	–	–	–	+	102.2 ± 4.9 (7)
3	–	–	–	+	–	101.4 ± 4.8 (3)
4	–	–	–	+	+	100.6 ± 5.7 (2)
5	+	–	–	–	–	119.8 ± 4.8 (5)
6	+	–	–	–	+	116.0 ± 3.6 (4)
7	–	+	–	–	–	105.3 ± 4.8 (4)
8	–	+	–	–	+	81.4 ± 9.3 (4)
9	–	–	+	–	–	105.2 ± 3.3 (8)
10	–	–	+	–	+	133.6 ± 4.3 (4)
11	+	+	–	–	–	117.3 ± 5.4 (4)
12	+	+	–	–	+	156.5 ± 3.7 (6)
13	+	–	+	–	–	116.1 ± 6.9 (8)
14	+	–	+	–	+	200.5 ± 9.3 (10)
15	+	+	+	–	+	202.1 ± 5.2 (3)
16	+	–	+	+	–	113.7 ± 6.1 (4)
17	+	–	+	+	+	213.8 ± 9.7 (4)

[a] Theophylline (2.5 mM) was always added to cyclic AMP

[b] Mean values (± SE) for the reaction velocity are expressed in percentages of the paired basal value (first line) and shown together with the number of individual experiments (in parentheses), the basal value averaging 175 ± 12 fmol/5 min per islet

phosphatidylserine and TPA, the reaction velocity at increasing concentrations of Ca^{2+} was compatible with a threshold concentration for activation by Ca^{2+} close to 0.04 μM and a K_a close to 5 μM (fig.1).

In order to further assess the specificity of the response to TPA, the experiments were repeated in an assay system designed to measure the activity of protein kinase A. In this system, cyclic AMP and theophylline, if used in combination, markedly increased the reaction velocity (table 2, lines 1–4). Such was not the case for the combination of phosphatidylserine, TPA and Ca^{2+} (table 2, line 5). The presence of the latter combination did not prevent the activation of the cyclic AMP-

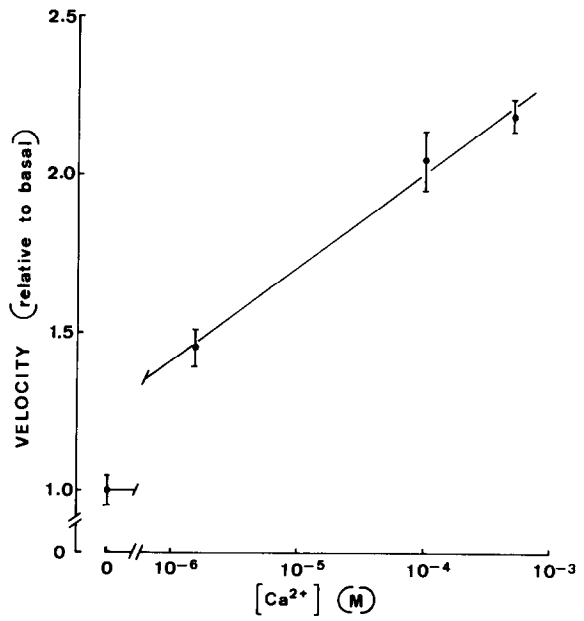

Fig.1. Effect of increasing Ca^{2+} concentrations (logarithmic scale) upon the velocity of histone phosphorylation by islet homogenates in the presence of phosphatidylserine (25 μg/ml) and TPA (20 nM). The basal value averaged 0.21 ± 0.02 pmol/5 min per islet. Mean values (± SE) refer to 6 individual determinations.

Table 2

Presence of cyclic AMP-dependent protein kinase in islet homogenates

Line	Phosphatidyl-serine (25 μg/ml)	TPA (20 nM)	Ca^{2+} (0.5 mM)	Cyclic AMP (10 μM)	Theophylline (2.5 mM)	Reaction velocity[a] (%)
1	−	−	−	−	−	100.0 ± 5.4 (10)
2	−	−	−	+	−	106.2 ± 2.8 (2)
3	−	−	−	−	+	108.4 ± 1.8 (2)
4	−	−	−	+	+	162.5 ± 5.3 (10)
5	+	+	+	−	−	91.4 ± 3.7 (3)
6	+	+	+	+	+	146.0 ± 9.4 (3)

[a] Mean values (± SE) for the reaction velocity are expressed in percentages of the mean basal value (first line) and shown together with the number of individual experiments (in parentheses), the basal value averaging 526 ± 32 fmol/10 min per islet

dependent kinase by the cyclic nucleotide and phosphodiesterase inhibitor (table 2, lines 5,6).

3.2. *TPA and the phosphatidylinositol cycle*

Two experimental approaches were used to detect a possible effect of TPA upon the turnover of inositol-containing phospholipids in the pancreatic islets. In the first approach, groups of 140 islets each were preincubated for 120 min in the presence of 2.8 mM D-glucose and a tracer amount of $^{32}P_i$ and then further incubated for 30 min at the same glucose and $^{32}P_i$ concentration in the absence or presence of TPA (2.0 μM). As shown in table 3, TPA failed to affect the ^{32}P-labelling of polyphosphoinositides, phosphatidylinositol, phosphatidic acid, phosphatidylethanolamine and lysophosphatidylcholine. However, the radioactivity associated with phosphatidylcholine was increased by about 50% above basal value ($p < 0.05$). In the second approach, groups of 150 islets each were preincubated for 180 min in the presence of 2.8 mM D-glucose and [^{3}H]inositol. The medium was then removed and the islets further incubated for 30 min in fresh medium containing 2.8 mM D-glucose, 10 mM LiCl, 1.0 mM unlabelled inositol and, as required, TPA (0.2 or 2.0 μM). The phorbol ester failed to stimulate the production of [^{3}H]inositol phosphates, which averaged 736 ± 72, 633 ± 21 and 822 ± 156 cpm/150 islets ($n = 3$ in each case) in the absence and presence of 0.2 μM and 2.0 μM TPA, respectively.

Table 3

Effect of TPA upon the radioactivity of phospholipids (cpm/islet) in islets exposed to either $^{32}P_i$ or [^{3}H]glycerol

TPA	$^{32}P_i$ labelling		[^{3}H]Glycerol labelling	
	Nil	2.0 μM	Nil	2.0 μM
PPI	53.6 ± 2.8[a]	51.9 ± 1.4	14.5 ± 0.7	13.4 ± 0.4
PA	26.2 ± 0.6	28.2 ± 1.8	6.7 ± 0.6	6.6 ± 0.3
PI	126.9 ± 4.7	129.2 ± 8.6	33.2 ± 2.4	31.7 ± 1.7
PC	34.7 ± 1.3	54.1 ± 2.5	73.0 ± 3.2	71.6 ± 4.8
LC	15.2 ± 2.9	16.9 ± 3.8	7.4 ± 1.1	6.7 ± 1.7
PE	34.8 ± 1.1	35.9 ± 1.8	90.7 ± 3.4	93.0 ± 2.4

[a] Mean values (± SE) refer to 3 individual observations in each case. PPI, polyphosphoinositides; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; LC, lysophosphatidylcholine; PE, phosphatidylethanolamine

Table 4

Effect of phospholipase C (10 munits/ml) upon insulin release

Line	D-Glucose (mM)	Ca^{2+} (mM)	Phospholipase C (source)	Insulin output (μunits/30 min per islet)			p
				Control	Test	Increment	
1	–	1.0	*C. perfringens*	8.9 ± 1.4 (17)	13.1 ± 1.3 (18)	+4.2 ± 1.9	<0.05
2	3.3	1.0	*C. perfringens*	9.0 ± 1.0 (34)	17.7 ± 1.8 (36)	+8.7 ± 2.1	<0.001
3	3.3	1.0	*B. cereus*	9.7 ± 1.5 (35)	34.6 ± 3.4 (36)	+24.9 ± 3.7	<0.001
4	3.3	–	*B. cereus*	11.4 ± 3.3 (18)	33.2 ± 2.1 (18)	+21.7 ± 3.9	<0.001
5	8.3	1.0	*B. cereus*	49.0 ± 2.4 (18)	74.1 ± 2.8 (18)	+25.0 ± 3.7	<0.001
6	16.7	1.0	*B. cereus*	113.9 ± 5.3 (18)	131.2 ± 5.7 (18)	+17.3 ± 7.7	<0.05

The finding that TPA increased the ^{32}P-labelling of phosphatidylcholine led us to perform two further series of experiments. In the first series, groups of 215 islets each were preincubated for 180 min in the presence of 2.8 mM D-glucose and a tracer amount of [^{3}H]glycerol, and then further incubated for 15 min at the same glucose and [^{3}H]glycerol concentrations in the absence or presence of TPA (2.0 μM). The phorbol ester failed to affect significantly the ^3H-labelling of islet phospholipids, including phosphatidylcholine and lysophosphatidylcholine (table 3). In the second series of experiments, groups of 60 islets each were preincubated for 120 min in the presence of 2.8 mM D-glucose and [^{3}H]choline, and then further incubated for 15 min at the same glucose concentration in the absence or presence of TPA (2.0 μM). The lipid-bound radioactivity was increased by TPA from 371 ± 24 to 478 ± 17 cpm/islet (n = 3 in each case, $p < 0.025$).

3.3. *Insulin output*

Phospholipase C (10 munits/ml) stimulated insulin release, whether in the absence or presence of D-glucose (table 2, lines 1,2). In this respect, the enzyme extracted from *B. cereus* was more potent than that from *C. perfringens* (table 4, lines 2,3). The absolute magnitude of the increment in the secretion rate was not significantly different at 3.3, 8.3 and 16.7 mM D-glucose, respectively (table 2, lines 3,5,6). When the islets were incubated for 30, 60 or 90 min at a low glucose concentration (3.3 mM), the phospholipase-induced increase in insulin output represented a fairly sustained phenomenon. Indeed, when expressed per min, the increment in the secretion rate after 90 min incubation was not significantly lower than the initial value enregistered during the first 30 min of incubation (paired ratio: 79.8 ± 19.2%; n = 18). When the islets were incubated in the absence of $CaCl_2$, the phospholipase-induced increase in insulin output was not significantly different from that seen in the presence of $CaCl_2$ (table 4, lines 3,4). At low glucose concentration, the secretory response to phospholipase C extracted from *B. cereus* was of a magnitude comparable to that evoked by 2.0 μM TPA. Thus, over 90 min incubation in the presence of 2.8 mM D-glucose, TPA (2.0 μM) augmented insulin output from a basal value of 16.6 ± 1.7 to 91.8 ± 4.5 μunits/90 min per islet (n = 40 in both cases).

4. DISCUSSION

The present results confirm the presence of protein kinase C activity in islet homogenates and reveal that in islets, like in other tissues [14,15], TPA can substitute for diacylglycerol as an enzyme activator. In the presence of TPA, the K_a of this kinase for Ca^{2+} was well suited to allow activation of the enzyme in response to physiological changes in cytosolic Ca^{2+} concentrations.

The direct activation of protein kinase C by TPA may account, in part at least, for the insulinotropic action of the phorbol ester [16–19]. Indeed, TPA failed to cause phosphoinositide breakdown in intact islets. The finding that TPA augmented the ^{32}P- and [^{3}H]choline-labelling of phosphatidylcholine, whilst failing to affect the incorporation of [^{3}H]glycerol in this phospholipid, indicates that TPA specifically enhanced the labelling of the polar head-group of phosphatidyl-

choline. A comparable effect of TPA was described in other cell types, e.g., in mouse ascites cells [20], and ascribed to stimulation of the reaction catalyzed by the enzyme CTP:phosphocholine cytidyltransferase [21].

The view that activation of protein kinase C may result in stimulation of insulin release is supported by the observation that phospholipase C provokes insulin release from intact islets [9]. In this respect, the reason why the enzyme extracted from *B. cereus* was more potent than that derived from *C. perfringens* is open to speculation. The present study reveals that exogenous phospholipase C stimulates insulin release both in the absence and presence of glucose. At glucose concentrations of 3.3 mM or more, the absolute magnitude of the increment in insulin output attributable to phospholipase C was little affected by the concentration of the hexose. The stimulation of insulin release by phospholipase C resembled that evoked by TPA in being a sustained phenomenon still detectable in media deprived of $CaCl_2$ [17]. The effect of phospholipase C could be due, therefore, to liberation of diglyceride from endogenous phospholipids and subsequent activation of protein kinase C.

Taken as a whole, the present data raise the idea that the activation of protein kinase C may account, in part at least, for the capacity of TPA to stimulate insulin release. This is not to deny that TPA also exerts modest but significant effects upon the handling of Ca^{2+} by the islet cells [17,18]. However, the knowledge that TPA fails to affect ^{45}Ca net uptake by the islets [17,18] and the finding that the secretory response to either TPA or exogenous phospholipase C is rather resistant to a shortage in extracellular Ca^{2+} suggest that activation of protein kinase C may stimulate insulin release even at close-to-basal cytosolic Ca^{2+} concentrations. A similar situation was recently reported in human platelets [22].

Several insulinotropic agents, including all nutrient secretagogues, stimulate the hydrolysis of phosphoinositides in the islet cells by a phospholipase C mechanism [11,12,23]. It is conceivable, therefore, that such a 'phosphatidylinositol effect' participates, via activation of protein kinase C and, possibly, other mechanisms [24], in the secretory response to these secretagogues. It could be argued that the secretory response to glucose differs from that evoked by TPA or exogenous phospholipase C in its greater sensitivity to a decrease in extracellular Ca^{2+} concentration. However, the effect of glucose to stimulate the hydrolysis of phosphoinositides is itself impaired in the absence of extracellular Ca^{2+} [12,23].

As our knowledge on the biophysical and biochemical organization of the pancreatic B-cell progresses, it becomes ever more evident that the secretory response of this cell to suitable secretagogues involves a great number of interconnected cellular events. This work emphasizes the view that protein kinase C may represent an essential component of this complex process of stimulus-secretion coupling.

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