

An inhibitory role for polyamines in protein kinase C activation and insulin secretion in mouse pancreatic islets

Peter THAMS, Kirsten CAPITO and Carl J. HEDESKOV

Department of Biochemistry A, University of Copenhagen, 3C Blegdamsvej, DK-2200 Copenhagen N, Denmark

The occurrence and function of polyamines in protein kinase C activation and insulin secretion in mouse pancreatic islets were studied. Determination of polyamines in mouse islets revealed 0.9 ± 0.3 (mean \pm s.e.m., $n = 6$) pmol of putrescine, 11.7 ± 3.2 (8) pmol of spermidine and 3.7 ± 0.6 (8) pmol of spermine per islet, corresponding to intracellular concentrations of 0.3–0.5 mM-putrescine, 3.9–5.9 mM-spermidine and 1.2–1.9 mM-spermine in mouse islets. Stimulation of insulin secretion by glucose, the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or the sulphonylurea glibenclamide did not affect these polyamine contents. In accordance with a role for protein kinase C in insulin secretion, TPA stimulated both protein kinase C activity and insulin secretion. Stimulation of insulin secretion by TPA was dependent on a non-stimulatory concentration of glucose and was further potentiated by stimulatory concentrations of glucose, glibenclamide or 3-isobutyl-1-methylxanthine, suggesting that protein kinase C activation, Ca^{2+} mobilization and cyclic AMP accumulation are all needed for full secretory response of mouse islets. Spermidine (5 mM) and spermine (1.5 mM) at concentrations found in islets inhibited protein kinase C stimulated by TPA + phosphatidylserine by 55% and 45% respectively. Putrescine (0.5 mM) was without effect, but inhibited the enzyme at higher concentrations (2–10 mM). Inhibition of protein kinase C by polyamines showed competition with Ca^{2+} , and Ca^{2+} influx in response to glucose or glibenclamide prevented inhibition of insulin secretion by exogenous polyamines at concentrations where they did not affect glucose oxidation. It is suggested that inhibition of protein kinase C by polyamines may be of significance for regulation of insulin secretion *in vivo* and that Ca^{2+} influx may function by displacing inhibitory polyamines bound to phosphatidylserine in membranes.

INTRODUCTION

An increase in the cytosolic concentration of Ca^{2+} is believed to trigger insulin release in the pancreatic β -cell, but details of the mechanism that produces this increase in cytosolic Ca^{2+} in response to physiological stimuli, and the means by which this second messenger initiates secretion, remain unknown. One phenomenon that seems to be important in the Ca^{2+} -mediated regulation of secretion is the phosphorylation of endogenous substrate proteins by Ca^{2+} -dependent protein kinases. Both Ca^{2+} -calmodulin-dependent and Ca^{2+} -phospholipid-dependent (protein kinase C) kinases have been identified in pancreatic islets (Gagliardino *et al.*, 1980; Tanigawa *et al.*, 1982), and islet homogenates have been found to exhibit Ca^{2+} -calmodulin-dependent and Ca^{2+} -phospholipid-dependent protein phosphorylation of endogenous proteins (Harrison & Ashcroft, 1982; Lord & Ashcroft, 1984; Thams *et al.*, 1984).

Protein kinase C is a Ca^{2+} - and phospholipid-dependent enzyme that is activated by diacylglycerol (Takai *et al.*, 1979; Kishimoto *et al.*, 1980). Diacylglycerol is transiently produced from inositol phospholipids in response to extracellular signals. Several reports suggest that a small amount of diacylglycerol dramatically increases the apparent affinity of protein kinase C for Ca^{2+} , activating the enzyme without any change in Ca^{2+} concentration (Kishimoto *et al.*, 1980; Kaibuchi *et al.*, 1981). The effect

of diacylglycerols on protein kinase C is mimicked by tumour-promoting phorbol esters such as TPA (Castagna *et al.*, 1982). Like diacylglycerol, TPA dramatically increases the affinity of the enzyme for Ca^{2+} , resulting in its activation without detectable cellular mobilization of Ca^{2+} (Yamanishi *et al.*, 1983; Rink *et al.*, 1983).

Several lines of evidence have implicated protein kinase C in regulation of insulin secretion. TPA has been found to stimulate insulin secretion from rat pancreatic islets (Virji *et al.*, 1978; Malaisse *et al.*, 1980). Furthermore, a marked synergism between TPA and the sulphonylurea gliclazide or the Ca^{2+} ionophore A23187 has been observed (Malaisse *et al.*, 1983; Zawalich *et al.*, 1983), suggesting that both protein kinase C activation and Ca^{2+} mobilization as described for other tissues (Nishizuka, 1984) are needed for the physiological response of pancreatic islets.

The synergism between intracellular Ca^{2+} mobilization and protein kinase C activation is usually attributed to the requirement for Ca^{2+} -calmodulin-mediated as well as protein kinase C-mediated phosphorylation (Nishizuka, 1984). This interpretation was originally devised for platelet activation (Castagna *et al.*, 1982). However, subsequent work revealed that Ca^{2+} ionophores do potentiate protein phosphorylation by protein kinase C in neutrophils and HL-60 cells (White *et al.*, 1984; May *et al.*, 1985).

A role for polyamines has recently been suggested in

stimulus-secretion coupling. Thus putrescine, spermidine and spermine at physiological concentrations have been found to inhibit the Ca^{2+} -calmodulin-dependent and Ca^{2+} -phospholipid-dependent protein kinases from several tissues (Qi *et al.*, 1983), suggesting that polyamines may be involved in the regulation of Ca^{2+} -dependent protein phosphorylation *in vivo*.

With the objective of assessing the role of protein kinase C in insulin secretion, the present study aimed at determining the function of polyamines. The results demonstrate the presence of substantial amounts of polyamines in mouse islets. The polyamines inhibit both TPA-induced insulin secretion and protein kinase C in a fashion that seems to be counteracted by stimulus-induced Ca^{2+} mobilization.

EXPERIMENTAL

Materials

Crude bacterial collagenase was obtained from Boehringer Mannheim, Germany, Tes, EGTA, benzamidine, dithiothreitol, phosphatidylserine, TPA, lysine-rich histone (type III-S, histone H1), putrescine, spermidine, spermine and ATP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Human serum albumin was from Behringswerke A.G., Marburg, Germany. Trifluoperazine was given by Rhône-Poulenc, Pharma Norden A/S, Copenhagen, Denmark. [γ - ^{32}P]ATP (sp. radioactivity 3000 Ci/mmol), [^{14}C]putrescine (118 mCi/mmol), [^{14}C]spermidine, (118 mCi/mmol), [^{14}C]spermine (118 mCi/mmol), [^{14}C]glucose (230 mCi/mmol), [^3H]inulin (1.6 Ci/mmol) and [^3H]dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride (10–30 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. ^{125}I -insulin and anti-(pig insulin) serum were kindly provided by Novo Industries A/S, Bagsvaerd, Denmark. All other chemicals were of analytical grade.

Preparation of islets

Islets were prepared by collagenase digestion (Coll-Garcia & Gill, 1969) of the pancreases of male albino mice (approx. 26 g body wt.) fed *ad libitum* on a standard laboratory diet.

Phosphorylation assay

A group of 500 mouse islets was collected in 100 μl of 25 mM-Tes buffer, pH 6.90, containing 5 mM- MgCl_2 , 1 mM-EGTA, 0.1 mM-dithiothreitol and 10 mM-benzamidine. A homogenate was prepared by sonication (10 s, 40 W). For the preparation of a soluble fraction, the homogenate was centrifuged for 20 min at 27000 g at 4 °C. The supernatant was frozen in liquid N_2 and stored at -80 °C until the next day.

Samples of islet cytosol (corresponding to 15–25 islets) were preincubated for 2 min in a reaction mixture (final volume 200 μl) containing 25 mM-Tes buffer, pH 6.90, 5 mM- MgCl_2 , 1 mM-EGTA, 0.1 mM-dithiothreitol, 10 mM- NaF , 10 mM-benzamidine and 0.2 mg of lysine-rich histone/ml, and further additions as indicated in the Figures and Tables. The reaction was started by addition of [γ - ^{32}P]ATP (500 c.p.m./pmol, final concn. 10 μM). The reaction was carried out at 30 °C for 2 min and terminated by transferring 150 μl of the incubation mixture to 1 ml of 25% (w/v) trichloroacetic acid

containing 0.3 mg of bovine albumin/ml as a carrier protein. The acid-precipitable radioactivity was then determined after three cycles of solubilization in 0.1 ml of 1 M- NaOH and precipitation with 2 ml of 25% trichloroacetic acid. The blank value, 0.35 ± 0.05 (5) pmol of [^{32}P]ATP, was not affected by polyamines. The enzyme activity, which was corrected for the activity in the absence of test agents, was linear as a function of the amount of islet protein and the incubation time for at least 5 min.

Polyamine assay

Groups of 100 islets were collected in 100 μl of Krebs-Henseleit (1932) buffer supplemented with 3.3 mM-glucose. After incubation for 30 min at 37 °C, 10 μl of test agents were added and the incubation proceeded for 5–60 min. The reaction was stopped by addition of 500 μl of ice-cold Krebs-Henseleit buffer, and the islets were collected by centrifugation and removal of medium. Polyamines were extracted by sonication (10 s, 40 W) in 100 μl of 0.2 M- HClO_4 . The resulting precipitates were removed by centrifugation at 6000 g for 10 min and the supernatants retained for polyamine determination.

The islet contents of putrescine, spermidine and spermine were measured by using a double-isotope derivative assay described by Paulus & Davis (1983). To 40 μl of islet extract were added 10 μl of a mixture containing 10 pmol of [^{14}C]putrescine (100 d.p.m./pmol), 100 pmol of [^{14}C]spermidine (100 d.p.m./pmol) and 50 pmol of [^{14}C]spermine (100 d.p.m./pmol) dissolved in water and 50 μl of [^3H]dansyl chloride (2 mg/ml, 0.5 mCi/ml) dissolved in acetone. After vigorous shaking, the mixture was made alkaline by addition of 10 mg of Na_2CO_3 and then vortex-mixed again. The tubes were sealed and incubated in the dark at room temperature overnight. After incubation, acetone was evaporated under a N_2 stream, and dansylated polyamines were extracted with 50 μl of toluene. This 20 μl of toluene extract was spotted on silica-gel thin-layer chromatograms. The chromatograms were developed in the dark in ethyl acetate/cyclohexane (2:2, v/v) in one dimension. After development the chromatograms were air-dried and the dansylated polyamines were detected by spraying the chromatograms with triethanolamine/propan-2-ol (1:4, v/v) and air-drying again.

The fluorescent spots were localized under u.v. light and scraped from the chromatograms and placed in 5 ml of scintillation fluid (Instagel II, Packard). After allowing 4 h for elution, the radioactivity was determined. The ^{14}C c.p.m. to ^3H c.p.m. ratio was determined after correction for channel overlap and background.

Assay of polyamine uptake

The uptake of [^{14}C]putrescine, [^{14}C]spermidine and [^{14}C]spermine by islets was assessed as follows. Groups of 25 islets were incubated at 37 °C in 50 μl of Krebs-Henseleit buffer with 3.3 mM-glucose and 10 mM [^{14}C]polyamine (0.5 Ci/mol) in micro-centrifuge tubes containing 20 μl of 1 M- HClO_4 , which was overlaid with 100 μl of silicone oil (sp. gr. 1.05). Incubations were terminated by centrifugation. The ends of the tubes were then cut off into counting vials containing 200 μl of water, and polyamines were extracted from the islets by sonication (10 s, 40 W) before addition of 1.2 ml of Instagel II (Packard) for scintillation counting. The inulin space, assumed to represent islet extracellular space, was

simultaneously determined by inclusion of [³H]inulin (3 μ M, 1.6 Ci/mmol) in the incubations and subtracted in the calculation of polyamine uptake.

Assay of glucose oxidation

Glucose oxidation was measured by determining the amount of CO₂ released from [U-¹⁴C]glucose, essentially as described by Ashcroft *et al.* (1970). Seven to ten islets were preincubated in the presence or absence of polyamines for 45 min in 15 μ l of Krebs–Henseleit buffer containing 3.3 mM-glucose in an atmosphere of O₂/CO₂ (19:1) before addition of 5 μ l of 56.9 mM-glucose in Krebs–Henseleit buffer containing polyamines and [U-¹⁴C]glucose (final sp. radioactivity 1.20 Ci/mol). After incubation for 2 h, 0.3 ml of Hyamine hydroxide was added to the glass beaker surrounding the incubation vessel to absorb released ¹⁴CO₂, and 30 μ l of 0.2 M-HCl was injected into the islet incubation medium to release trapped ¹⁴CO₂. After incubation at room temperature overnight, the radioactivity absorbed by the Hyamine hydroxide was determined by liquid-scintillation counting. The blank value in the absence of islets, 189 \pm 17 (3) pmol of [¹⁴C]glucose after 2 h of incubation, was not affected by polyamines.

Insulin assay

Insulin release from islets was measured in batch-type incubations. Batches of five or six medium-sized islets were transferred to test tubes containing 600 μ l of Krebs–Henseleit buffer with 2 mg of human serum albumin/ml and 3.3 mM-glucose. After preincubation for 45 min at 37 °C, the medium was replaced by 600 μ l of the same medium also containing test agents as indicated in the Tables and Figures, and the islets were incubated for 2 h at 37 °C. When added, polyamines were present during both the preincubation and the incubation period. The polyamines were added to the medium without correction for osmolarity, but, when necessary, the pH of the medium was adjusted to pH 7.40 after addition of test substance. After incubation, samples of the incubation medium were diluted with 0.04 M-phosphate buffer,

pH 7.40, containing 1 mg of human serum albumin/ml and stored at –20 °C until assayed by a radioimmunoassay (Heding, 1966) with rat insulin as standard.

TPA was added in a small volume of dimethyl sulphoxide, final concn. 0.01–0.1% (v/v). At these low concentrations, dimethyl sulphoxide did not affect insulin secretion or any other parameter assayed. Results are given as means \pm S.E.M. (*n* = number of experiments).

RESULTS

Effect of TPA on insulin secretion

TPA did not stimulate secretion in the absence of glucose, but its effect was dependent on a sub-stimulatory concentration of glucose (3.3 mM) (Table 1); 0.16 μ M-TPA was a maximum stimulating concentration at 3.3 mM-glucose (Table 1). Glucose (16.7 mM), which stimulates Ca²⁺ influx and cyclic AMP accumulation in islets (Hedeskov, 1980), potentiated insulin secretion by TPA (Table 1). Thus TPA at 3.3 mM-glucose stimulated the release of approx. 700 μ units of insulin/five islets per 2 h, whereas the release attributable to TPA in the presence of 16.7 mM glucose was approx. 1800 μ units of insulin/five islets per 2 h (*P* < 0.05). Likewise, the sulphonylurea glibenclamide (20 μ M), which stimulates Ca²⁺ influx in islets (Gylfe *et al.*, 1984), or the methylxanthine IBMX (1 mM), which causes a large increase in cyclic AMP in islets (Christie & Ashcroft, 1984), potentiated insulin secretion by TPA from 800 to 1700 (*P* < 0.05) and 2900 (*P* < 0.005) μ units/five islets per 2 h respectively (Table 1). Trifluoperazine (25 μ M), which previously has been shown to inhibit insulin secretion evoked by a number of agents, including glucose (Sugden *et al.*, 1979; Janjic *et al.*, 1981; Henquin, 1981), and to inhibit Ca²⁺-calmodulin-dependent protein kinase and protein kinase C in mouse islets (Thams *et al.*, 1984), inhibited insulin release by TPA to 57% of the value in the absence of trifluoperazine (Table 1).

Table 1. Effect of TPA on insulin secretion

Insulin secretion was measured as described in the Experimental section. Results are means \pm S.E.M. with the numbers of experiments given in parentheses. Statistical evaluation of the data was made by a paired *t* test: NS, not significant (*P* > 0.05).

Line	Added agents					Insulin release (μ units/five islets/2 h)	<i>P</i>
	Glucose (mM)	TPA (μ M)	Glibenclamide (μ M)	IBMX (mM)	Trifluoperazine (μ M)		
1	–	–	–	–	–	717 \pm 124 (6)	
2	–	0.16	–	–	–	853 \pm 133 (6)	Versus 1: NS
3	3.3	–	–	–	–	513 \pm 114 (5)	
4	3.3	0.16	–	–	–	1199 \pm 216 (5)	Versus 3: < 0.05
5	3.3	1.6	–	–	–	1312 \pm 104 (5)	Versus 3: < 0.001; versus 4: NS
6	16.7	–	–	–	–	1910 \pm 322 (4)	Versus 3: < 0.01
7	16.7	0.16	–	–	–	3788 \pm 524 (4)	Versus 4: < 0.005; versus 6: < 0.005
8	3.3	–	20	–	–	756 \pm 108 (4)	Versus 3: < 0.01
9	3.3	1.6	20	–	–	2406 \pm 319 (4)	Versus 5: < 0.05; versus 8: < 0.02
10	3.3	–	–	1	–	669 \pm 45 (3)	Versus 3: NS
11	3.3	1.6	–	1	–	3701 \pm 560 (3)	Versus 5: < 0.005; versus 10: < 0.001
12	3.3	–	–	–	25	416 \pm 110 (3)	Versus 3: NS
13	3.3	1.6	–	–	25	744 \pm 37 (4)	Versus 5: < 0.05

Table 2. Polyamine contents in mouse pancreatic islets

Polyamines were determined as described in the Experimental section. Results are means \pm S.E.M. for the numbers of experiments in parentheses. '3.3–16.7' mM glucose represents the pooled data for 3.3 mM- and 16.7 mM-glucose. The significance of the differences from control was assessed by using Student's *t* test. N.D., not determined.

Added agents			Incubation time (min)	Polyamine content (pmol/islet)		
Glucose (mM)	TPA (μ M)	Glibenclamide (μ M)		Putrescine	Spermidine	Spermine
3.3	–	–	5	0.9 \pm 0.3 (6)	11.7 \pm 3.2 (8)	3.7 \pm 0.6 (8)
16.7	–	–	5	0.8 \pm 0.2 (5)	11.8 \pm 3.2 (8)	4.7 \pm 0.8 (8)
3.3	–	–	60	0.6 \pm 0.3 (5)	6.3 \pm 1.1 (11)	4.0 \pm 0.5 (11)
16.7	–	–	60	0.5 \pm 0.2 (5)	6.7 \pm 1.3 (5)	3.9 \pm 0.8 (5)
'3.3–16.7'	–	–	5	0.8 \pm 0.2 (11)	11.7 \pm 1.9 (16)	4.2 \pm 0.5 (16)
'3.3–16.7'	–	–	60	0.6 \pm 0.1 (10)	6.4 \pm 0.8 (16)*	4.0 \pm 0.4 (16)
3.3	1.6	–	60	N.D.	6.4 \pm 1.3 (3)	3.9 \pm 1.3 (3)
3.3	–	20	60	N.D.	5.6 \pm 1.0 (4)	3.0 \pm 0.6 (4)†
3.3	1.6	20	60	N.D.	5.1 \pm 0.8 (4)	2.7 \pm 0.3 (4)

**P* < 0.02, versus '3.3–16.7' mM-glucose, 5 min of incubation; non-paired data.

†*P* < 0.05, versus 3.3 mM-glucose, 60-min of incubation; paired data.

Polyamine contents in mouse islets

Determination of polyamines in mouse islets revealed 0.9 pmol of putrescine, 11.7 pmol of spermidine and 3.7 pmol of spermine per islet (Table 2). Assuming equal distribution in the intracellular water space and a total water space of approx. 2–3 nl/islet (Ashcroft *et al.*, 1970; Sener & Malaisse, 1978), these values correspond to 0.3–0.5 mM-putrescine, 3.9–5.9 mM-spermidine and 1.2–1.9 mM-spermine in mouse islets. Glucose did not affect these values as measured after 5 or 60 min of incubation (Table 2). However, the concentration of spermidine appeared to decrease with incubation time irrespective of the glucose concentration present. Thus spermidine decreased from 11.7 pmol/islet after 5 min of incubation to 6.4 pmol/islet after 60 min of incubation, or by approx. 45% (*P* < 0.02). The nature of this decrease remains to be established.

TPA has been described to induce ornithine decarboxylase, the key enzyme in polyamine synthesis (Koenig *et al.*, 1983; Cope *et al.*, 1984) or to stimulate spermidine/spermine *N*¹-acetyltransferase, the rate-limiting enzyme in polyamine degradation (Matsui-Yuasa *et al.*, 1984). However, TPA did not affect the contents of spermidine and spermine in islets as measured after 60 min of incubation (Table 2). On the other hand, glibenclamide or glibenclamide+TPA did show a tendency to decrease the concentration of spermine, by approx. 21% (*P* < 0.05) and 29% (*P* < 0.2) respectively (Table 2).

Polyamine uptake by mouse islets

In the presence of 10 mM-putrescine, -spermidine or -spermine in the incubation medium, mouse islets accumulated polyamines during a 165 min incubation (Fig. 1). Putrescine and spermine accumulated to approx. 16 pmol/islet during incubation, whereas the uptake of spermidine was restricted to approx. 7 pmol/islet after 165 min of incubation, probably reflecting the high endogenous content of this polyamine in mouse islets. Taking into account the endogenous content of polyamines in islets (Table 2), these values correspond to a

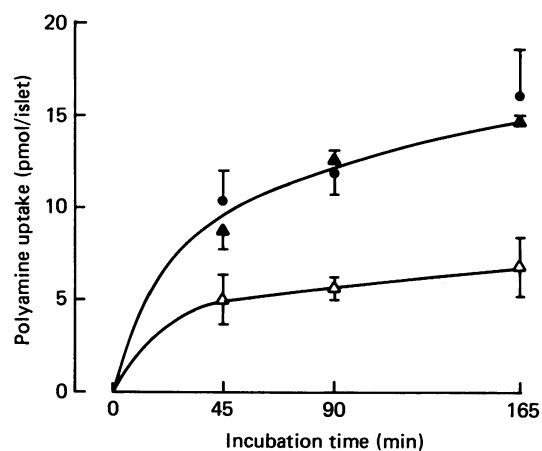


Fig. 1. Time course of polyamine uptake by mouse islets

Uptake of putrescine (●), spermidine (△) and spermine (▲) was determined as described in the Experimental section at a concentration of 10 mM of each polyamine in the medium. Results are means \pm S.E.M. (*n* = 3).

total content of 17 pmol of putrescine, 20 pmol of spermine and 19 pmol of spermidine per islet, or an intracellular concentration of approx. 6–10 mM of each polyamine after 165 min of incubation.

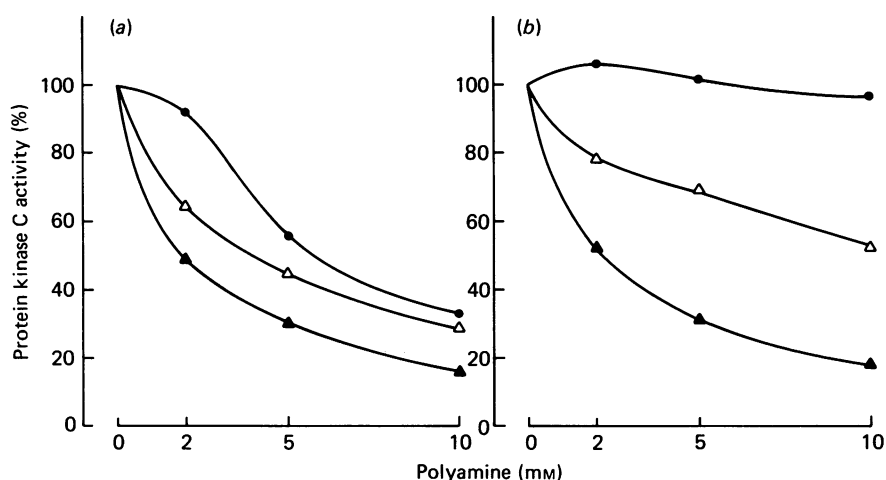
Effect of polyamines on protein kinase C

In a previous paper we described the presence of protein kinase C and a large number of endogenous substrate proteins for this kinase in mouse islets (Thams *et al.*, 1984). Table 3 describes the effect of TPA on this activity. Full activation of the enzyme was achieved by addition of Ca²⁺ + TPA + phosphatidylserine. The stimulation by TPA was greatly dependent on phosphatidylserine. Thus TPA + phosphatidylserine in the absence of Ca²⁺ stimulated phosphorylation to 94% of the activity in the presence of all three stimulators, or to the same degree as Ca²⁺ + phosphatidylserine in the absence

Table 3. Effect of TPA on protein kinase C activity

Protein kinase activity was determined as described in the Experimental section. Results are given as mean percentages (\pm S.E.M. for the numbers of experiments in parentheses) of the activity in the presence of Ca^{2+} ($20 \mu\text{M}$) + TPA (32 nM) + phosphatidylserine (PS; $20 \mu\text{g/ml}$) [0.57 ± 0.07 (10) pmol/islet per 2 min]. PS was suspended in 25 mM-Tes buffer, pH 6.9, by sonication (1 min, 40 W) at 0°C before use in the assay. EGTA (1 mM) was included in all cases; 1 mM-CaCl_2 was added to obtain a free Ca^{2+} concentration of $20 \mu\text{M}$. The significance of the difference from the value in the presence of Ca^{2+} ($20 \mu\text{M}$) + TPA (32 nM) + PS ($20 \mu\text{g/ml}$) was assessed by using a paired *t* test: NS, not significant ($P > 0.05$).

Added agents	Activity (%)	<i>P</i>
Ca^{2+} ($20 \mu\text{M}$) + TPA (32 nM) + PS ($20 \mu\text{g/ml}$)	100 ± 12 (10)	
TPA (32 nM) + PS ($20 \mu\text{g/ml}$)	94 ± 12 (10)	NS
Ca^{2+} ($20 \mu\text{M}$) + PS ($20 \mu\text{g/ml}$)	94 ± 6 (5)	NS
Ca^{2+} ($20 \mu\text{M}$) + TPA (32 nM)	24 ± 5 (3)	< 0.005
TPA (32 nM)	25 ± 4 (3)	< 0.001
PS ($20 \mu\text{g/ml}$)	11 ± 2 (4)	< 0.001
Ca^{2+} ($20 \mu\text{M}$)	7 ± 2 (8)	< 0.001

**Fig. 2. Effects of polyamines on protein kinase C activity**

The effect of increasing concentrations of putrescine (●), spermidine (Δ) and spermine (▲) on protein kinase C activated by (a) TPA (32 nM) + phosphatidylserine ($20 \mu\text{g/ml}$) or (b) Ca^{2+} ($20 \mu\text{M}$) + TPA (32 nM) + phosphatidylserine ($20 \mu\text{g/ml}$) was determined as described in the Experimental section. EGTA (1 mM) was included in all cases; 1 mM-CaCl_2 was added to obtain a free Ca^{2+} concentration of $20 \mu\text{M}$. Results are means of two experiments run in triplicate and are given as percentages of the activity in the absence of polyamines.

of TPA. In comparison, Ca^{2+} + TPA in the absence of added phosphatidylserine only stimulated phosphorylation to 24% of that with the fully activated enzyme (Table 3). These results are consistent with previous reports for other tissues, demonstrating a dramatic decrease in Ca^{2+} requirement for protein kinase C activation in the presence of diacylglycerol or tumour-promoting phorbol esters (Kaibuchi *et al.*, 1981; Yamanishi *et al.*, 1983).

Putrescine, spermidine and spermine inhibited TPA + phosphatidylserine-induced protein kinase C activity in a dose-dependent manner (Fig. 2). At the concentrations found in islets, putrescine (0.5 mM) did not inhibit, whereas spermidine (5 mM) and spermine (1.5 mM) inhibited the TPA + phosphatidylserine-activated enzyme to 45% and 55% respectively of the activity observed in the absence of polyamines (Fig. 2).

Addition of Ca^{2+} abolished inhibition of protein kinase C by putrescine and decreased inhibition by spermidine,

but did not affect inhibition by spermine (Fig. 2), suggesting that at least putrescine and spermidine interfere with Ca^{2+} -sensitive sites involved in stimulation of the enzyme. Inhibition by spermidine could not be totally prevented by Ca^{2+} , and an increase in Ca^{2+} from 20 to $100 \mu\text{M}$, in TPA from 32 to 320 nM or in phosphatidylserine from 20 to $100 \mu\text{g/ml}$ did not relieve inhibition by spermidine (5 mM) observed with Ca^{2+} ($20 \mu\text{M}$) + TPA (32 nM) + phosphatidylserine ($20 \mu\text{g/ml}$) (results not shown).

Effect of polyamines on insulin secretion

To examine the possible significance of polyamines in protein kinase C activation, the effects of exogenous polyamines on insulin secretion were investigated. In accordance with the observed order of potency for polyamines as inhibitors of protein kinase C, putrescine (10 mM), spermidine (10 mM) and spermine (10 mM) inhibited TPA-induced insulin secretion by 40%, 68%

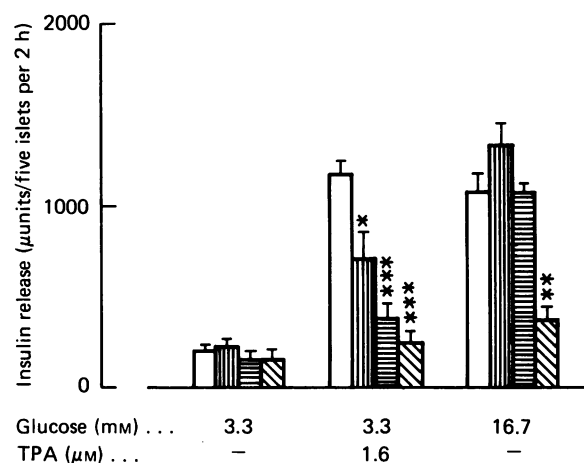


Fig. 3. Effects of polyamines on TPA-induced and glucose-induced insulin secretion

Insulin secretion was measured as described in the Experimental section without (□) or with 10 mM-putrescine (▨), 10 mM-spermidine (▩) or 10 mM spermine (▧) and further additions as indicated. Results are means \pm S.E.M. ($n = 4-22$). The significance of the differences from controls in the absence of polyamines was assessed by using a paired t test: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

and 79% respectively (Fig. 3). Likewise a 65% inhibition of glucose-stimulated secretion by spermine (10 mM) could be demonstrated. In contrast, however, both putrescine (10 mM) and spermidine (10 mM) failed to inhibit glucose-induced insulin secretion.

To investigate whether Ca^{2+} influx or cyclic AMP accumulation in response to glucose may counteract inhibition of protein kinase C by polyamines, the effects of glibenclamide and IBMX on TPA-induced insulin secretion in the presence of spermidine (10 mM) and spermine (10 mM) were investigated (Fig. 4). Spermidine did not inhibit glibenclamide-induced or IBMX-induced insulin secretion. Addition of glibenclamide totally abolished spermidine inhibition of TPA-induced insulin secretion. IBMX, on the other hand, could not prevent inhibition by spermidine of TPA-induced secretion. Spermine (10 mM) inhibited glibenclamide-induced, IBMX-induced, glibenclamide + TPA-induced and IBMX + TPA-induced secretion by 36%, 27%, 74% and 79% respectively (Fig. 4).

Effects of polyamines on glucose oxidation

To examine the effect of polyamines on islet oxidative metabolism, the effect of polyamines on glucose oxidation was investigated. Table 4 shows that the amines displayed varied effects on $^{14}CO_2$ release from [U- ^{14}C]glucose when tested at the concentrations used in the

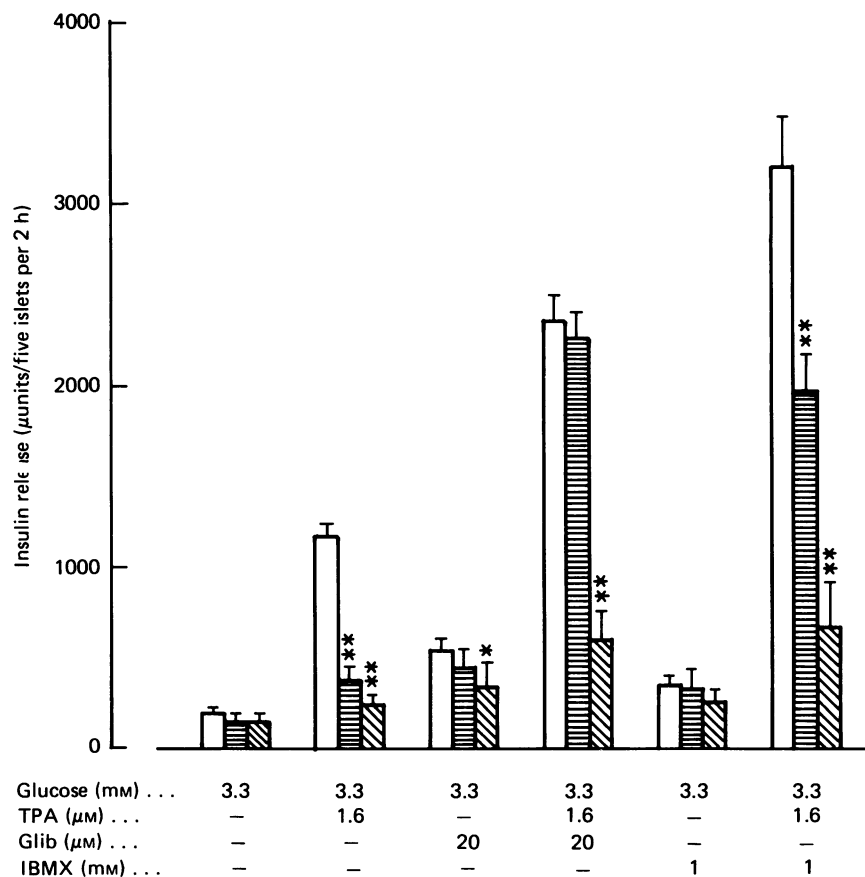


Fig. 4. Effects of glibenclamide and IBMX on TPA-induced insulin secretion in the presence of polyamines

Insulin secretion was measured as described in the Experimental section without (□) or with 10 mM-spermidine (▩) or 10 mM-spermine (▧) and further additions as indicated. Results are means \pm S.E.M. ($n = 4-18$). The significance of the differences from controls in the absence of polyamines was assessed by a paired t test: * $P < 0.05$; ** $P < 0.001$. Abbreviation: Glib, glibenclamide.

Table 4. Effects of polyamines on glucose oxidation

Oxidation of [U-¹⁴C]glucose to ¹⁴CO₂ was determined at 16.7 mM-glucose as described in the Experimental section. Results are given as mean percentages (\pm S.E.M. for the numbers of experiments in parentheses) of the activity in the absence of polyamines [726 \pm 172 (3) pmol/10 islets per 2 h]. Statistical evaluation of the data was made by a paired *t* test: NS, not significant (*P* > 0.05).

Polyamine added (10 mM)	Activity (%)	<i>P</i>
—	100	
Putrescine	101 \pm 20 (3)	NS
Spermidine	102 \pm 10 (3)	NS
Spermine	40 \pm 4 (3)	< 0.001

experiments on insulin release. Spermine at 10 mM was a potent inhibitor of glucose oxidation, causing a 60% inhibition at 16.7 mM-glucose. Putrescine (10 mM) and spermidine (10 mM), on the other hand, failed to inhibit glucose oxidation.

DISCUSSION

The results demonstrate that protein kinase C may play a role in insulin secretion. Thus TPA stimulated both protein kinase C and insulin secretion, and both responses were inhibited by the polyamines putrescine and spermidine at concentrations where these polyamines did not affect glucose oxidation. Spermine also inhibited protein kinase C and insulin secretion, but showed additional effects on glucose oxidation at the concentration used, suggesting that this polyamine, at least at 10 mM, is not specifically inhibiting protein kinase C. We have, however, not attempted to correlate the inhibition of protein kinase C and insulin secretion by polyamines. This cannot be done for three reasons: (a) the inhibition by polyamines was sensitive to Ca²⁺, (b) the exact cytosolic concentrations of Ca²⁺ during basal and stimulated conditions are not known, and (c) endogenous polyamines in the islets may contribute to the overall response.

At variance with previous results from rat pancreatic islets (Malaisse *et al.*, 1980; Zawalich *et al.*, 1983), mouse islets did not respond to TPA in the absence of glucose, which may suggest that activation of protein kinase C alone does not lead to insulin secretion. The secretory response to TPA was dependent on a non-stimulatory concentration of glucose, and further potentiated by stimulatory concentrations of glucose, glibenclamide or IBMX, which are known to stimulate Ca²⁺ fluxes and cyclic AMP accumulation in islets (Hedeskov, 1980; Gylfe *et al.*, 1984). This is in agreement with previous suggestions that Ca²⁺ mobilization, cyclic AMP accumulation and protein kinase C activation are needed for the full physiological response of pancreatic islets (Zawalich *et al.*, 1983).

The findings that putrescine and spermidine did not inhibit glucose-induced insulin secretion do not rule out an involvement of protein kinase C in glucose-induced secretion, but rather suggest that glucose abolishes inhibition of protein kinase C by these polyamines. The ability of glibenclamide to prevent inhibition of TPA-

induced secretion by spermidine and the observed competition between Ca²⁺ and polyamines on protein kinase C suggest that this effect of glucose may be attributed to an influx of Ca²⁺. A possible contribution by cyclic AMP, on the other hand, cannot be excluded, since IBMX apparently decreased inhibition by spermidine. However, since IBMX and cyclic AMP may have effects on cellular Ca²⁺ (Henquin, 1985), this effect similarly may reflect a mobilization of Ca²⁺.

The most obvious specific characteristics of polyamines are their polybasic character, which gives them a high affinity for acidic constituents, and it is likely that the polybasic nature of polyamines is important in determining their physiological action. Their mechanism of action is still unknown. In dealing with protein kinases, a diversity of effects have been described in several tissues. Thus polyamines have been shown to stimulate cyclic nucleotide-independent protein kinases (casein kinase II) (Mäenpää, 1977; Yamamoto *et al.*, 1979), to be without effect on cyclic-nucleotide-dependent protein kinases (Qi *et al.*, 1983), or to inhibit Ca²⁺-dependent protein kinases (Qi *et al.*, 1983). Quite different mechanisms of action may be operative in these enzyme-polyamine interactions, although the relative order of potency of the polyamines (i.e. spermine > spermidine > putrescine) is the same for both inhibition and stimulation of these kinases, in accordance with the polybasic nature determining their physiological action. Although the exact mechanism by which phosphatidylserine activates protein kinase C is not known, neutralization of the negative charges on phosphatidylserine by the positively charged polyamines, probably competing with Mg²⁺ and Ca²⁺ for binding sites, may form the basis for the inhibitory effects of these agents. It is tempting to speculate that polyamines bound to phosphatidylserine in membranes prevent binding and activation of protein kinase C, and that Ca²⁺ influx functions by displacing membrane-bound polyamines. The findings that Ca²⁺ decreased inhibition of protein kinase C by putrescine and spermidine substantiate this possibility. Previous work has demonstrated that spermine and to a lesser extent spermidine, but not putrescine, may cause aggregation of phosphatidylserine liposomes (Schuber *et al.*, 1983), and it is possible that the apparently non-competitive inhibition of protein kinase C by spermine and in part by spermidine *in vitro* is due to aggregation of the liposomes on binding of these polyamines.

The occurrence of high contents of polyamines in mouse islets, in accordance with results from rat islets (Bungay *et al.*, 1984), suggests that they may play a role in insulin secretion. Thus the concentrations of spermidine and spermine are high enough for them to cause inhibition of protein kinase C, and the dependency on Ca²⁺ for TPA-induced insulin secretion may at least in part be explained by Ca²⁺ relieving inhibition by polyamines of this kinase.

The synergism between intracellular Ca²⁺ mobilization and protein kinase C activation is usually attributed to the requirement for Ca²⁺-calmodulin-mediated as well as protein kinase C-mediated phosphorylation (Nishizuka, 1984). Previous work from several laboratories has implicated Ca²⁺-calmodulin-dependent and cyclic AMP-dependent protein phosphorylation in insulin secretion (Harrison & Ashcroft, 1982; Thams *et al.*, 1984; Christie & Ashcroft, 1984). The present results, however, cast some doubt as to the relative importance of Ca²⁺-

calmodulin-dependent protein phosphorylation in insulin secretion and suggest that elevated Ca^{2+} concentrations have a synergistic effect with diacylglycerol on protein kinase C activation. So far the combined effect of Ca^{2+} and TPA on protein kinase C-dependent protein phosphorylation in intact islets has not been investigated. However, investigations in other tissues have demonstrated that Ca^{2+} does synergize protein phosphorylation by protein kinase C in whole cells (White *et al.*, 1984; May *et al.*, 1985).

The present findings have clearly demonstrated the occurrence of high concentrations of the polyamines putrescine, spermidine and spermine in mouse pancreatic islets. The involvement of protein kinase C in insulin secretion is substantiated by the findings that TPA stimulates both protein kinase C and insulin secretion and that both responses are inhibited by polyamines. The concentrations of polyamines found in islets suggest that both spermidine and spermine may inhibit protein kinase C *in vivo* and the physiological significance of polyamines is strengthened by the findings that Ca^{2+} influx prevents inhibition by polyamines of insulin secretion. The secretory response to TPA is dependent on Ca^{2+} , which may implicate Ca^{2+} -calmodulin-dependent protein kinase, but also suggests that Ca^{2+} may be needed for the displacement of polyamines which are bound to phosphatidylserine in membranes and thus prevent activation of protein kinase C.

This work was supported by financial aid from the Danish Medical Research Council (grant no. 12-5693), Novo's Foundation, Nordic Insulin Foundation, P. Carl Petersen's Foundation, Dr. med. Erik Garde and Elisabeth Garde's legat and the Danish Diabetic Association. The skillful technical assistance of Mrs. Ingrid Mikkelsen and Mr. Peter V. Larsen is highly appreciated.

REFERENCES

- Ashcroft, S. J. H., Hedekov, C. J. & Randle, P. J. (1970) *Biochem. J.* **118**, 143–154
- Bungay, P. J., Potter, J. M. & Griffin, M. (1984) *Biosci. Rep.* **4**, 869–877
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Christie, M. R. & Ashcroft, S. J. H. (1984) *Biochem. J.* **218**, 87–89
- Coll-Garcia, E. & Gill, J. R. (1969) *Diabetologia* **5**, 61–66
- Cope, F. O., Conrad, E. A., Staller, J. M. & Boutwell, R. K. (1984) *Cancer Lett.* **23**, 331–342
- Gagliardino, J. J., Harrison, D. E., Christie, M. R., Gagliardino, E. E. & Ashcroft, S. J. H. (1980) *Biochem. J.* **192**, 919–927
- Gylfe, E., Hellman, B., Sehlin, J. & Täljedal, I.-B. (1984) *Experientia* **40**, 1126–1134
- Harrison, D. E. & Ashcroft, S. J. H. (1982) *Biochim. Biophys. Acta* **714**, 313–319
- Hedekov, C. J. (1980) *Physiol. Rev.* **60**, 442–509
- Heding, L. G. (1966) in *Labelled Proteins in Tracer Studies* (Donato, L., Milhaud, G. & Sirdus, J., eds.), pp. 345–350, Euratom, Brussels
- Henquin, J. C. (1981) *Biochem. J.* **196**, 771–780
- Henquin, J. C. (1985) *Arch. Int. Physiol. Biochim.* **93**, 37–48
- Janjic, D., Wollheim, C. B., Siegel, E. G., Krausz, Y. & Sharp, G. W. G. (1981) *Diabetes* **30**, 960–966
- Kaibuchi, K., Takai, Y. & Nishizuka, Y. (1981) *J. Biol. Chem.* **256**, 7146–7149
- Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 2273–2276
- Koenig, H., Goldstone, A. D. & Lu, C. Y. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7210–7214
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Lord, J. M. & Ashcroft, S. J. H. (1984) *Biochem. J.* **219**, 547–551
- Mäenpää, P. H. (1977) *Biochim. Biophys. Acta* **498**, 294–305
- Malaisse, W. J., Sener, A., Herchuelz, A., Carpinelli, A. R., Poloczek, P., Winand, J. & Castagna, M. (1980) *Cancer Res.* **40**, 3827–3831
- Malaisse, W. J., Lebrun, P., Herchuelz, A., Sener, A. & Malaisse-Lagae, F. (1983) *Endocrinology (Baltimore)* **113**, 1870–1877
- Matsui-Yuasa, I., Otani, S., Shu, Z. W. & Morisawa, S. (1984) *FEBS Lett.* **178**, 297–300
- May, W. S., Sahyoun, N., Wolf, M. & Cuatrecasas, P. (1985) *Nature (London)* **317**, 549–551
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Paulus, T. J. & Davis, R. H. (1983) *Methods Enzymol.* **94**, 36–42
- Qi, D.-F., Schatzman, R. C., Mazzei, G. J., Turner, R. S., Raynor, R. L., Liao, S. & Kuo, J. F. (1983) *Biochem. J.* **213**, 281–288
- Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) *Nature (London)* **305**, 317–319
- Schuber, F., Hong, K., Düzgünes, N. & Papahadjopoulos, D. (1983) *Biochemistry* **22**, 6134–6140
- Sener, A. & Malaisse, W. J. (1978) *Diabete Metab.* **4**, 127–133
- Sugden, M. C., Christie, M. R. & Ashcroft, S. J. H. (1979) *FEBS Lett.* **105**, 95–100
- Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. & Nishizuka, Y. (1979) *Biochem. Biophys. Res. Commun.* **91**, 1218–1224
- Tanigawa, K., Kuzuya, H., Imura, H., Taniguchi, H., Baba, S., Takai, Y. & Nishizuka, Y. (1982) *FEBS Lett.* **138**, 183–186
- Thams, P., Capito, K. & Hedekov, C. J. (1984) *Biochem. J.* **221**, 247–253
- Virji, M. A. G., Steffes, M. W. & Estensen, R. D. (1978) *Endocrinology (Baltimore)* **102**, 706–711
- White, J. R., Huang, C.-K., Hill, J. M., Naccache, P. H., Becker, E. L. & Sha'afi, R. I. (1984) *J. Biol. Chem.* **259**, 8605–8611
- Yamamoto, M., Criss, W. E., Takai, Y., Yamamura, H. & Nishizuka, Y. (1979) *J. Biol. Chem.* **254**, 5049–5052
- Yamanishi, J., Takai, Y., Kaibuchi, K., Sano, K., Castagna, M. & Nishizuka, Y. (1983) *Biochem. Biophys. Res. Commun.* **112**, 778–786
- Zawalich, W., Brown, C. & Rasmussen, H. (1983) *Biochem. Biophys. Res. Commun.* **117**, 448–455

Received 17 January 1986/24 February 1986; accepted 3 March 1986