Phorbol-ester-induced down-regulation of protein kinase C in mouse pancreatic islets

Potentialiation of phase 1 and inhibition of phase 2 of glucose-induced insulin secretion

Peter THAMS,*† Kirsten CAPITO,* Carl J. HEDESKOV* and Hans KOFOD†

*Department of Biochemistry A, University of Copenhagen, 3C Blegdamsvej, DK-2200 Copenhagen N, and †Hagedorn Research Laboratory, 6 Niels Steensensvej, DK-2820 Gentofte, Denmark

The influence of down-regulation of protein kinase C on glucose-induced insulin secretion was studied. A 22–24 h exposure of mouse pancreatic islets to the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 0.16 μM) in RPMI 1640 culture medium (8.3 mM-glucose, 0.43 mM- Ca^{2+}) abolished TPA (0.16 μM)-induced insulin secretion and led to a potentiation of phase 1 and a decrease in phase 2 of glucose-induced insulin secretion. Thus, although the total insulin release during 40 min of perfusion with glucose (16.7 mM) (45–85 min) was unaffected, the percentage released during phase 1 (45–55 min) was increased from 12.9 ± 1.5 (4)% in controls to 35.8 ± 3.9 (4)% in TPA-treated islets ($P < 0.01$), and the percentage released during phase 2 (65–85 min) was decreased from 63.2 ± 3.9 (4)% to 35.3 ± 1.4 (4)% ($P < 0.005$). In contrast, TPA exposure in TCM 199 medium (5.5 mM-glucose, 1.26 mM- Ca^{2+}) caused a total abolition of both phases 1 and 2 of glucose-induced secretion. However, inclusion of the α_2 -adrenergic agonists adrenaline (10 μM) or clonidine (10 μM), or lowering of the Ca^{2+} concentration in TCM 199 during down-regulation, preserved and potentiated phase 1 of glucose-induced secretion. Furthermore, perfusion of islets in the presence of staurosporine (1 μM), an inhibitor of protein kinase C, potentiated phase 1 and inhibited phase 2 of glucose-induced secretion. In addition, down-regulation of protein kinase C potentiated phase 1 and inhibited phase 2 of carbamoylcholine (100 μM)-induced insulin secretion at 3.3 mM-glucose, and abolished the potentiating effect of carbamoylcholine (100 μM) at 16.7 mM-glucose. These results substantiate a role for protein kinase C in insulin secretion, and suggest that protein kinase C inhibits phase 1 and stimulates phase 2 of both glucose-induced and carbamoylcholine-induced insulin secretion.

INTRODUCTION

Recent evidence suggests that glucose-induced insulin secretion from the pancreatic β -cell may be controlled by three convergent and interdependent regulatory pathways involving changes in the cytosolic Ca^{2+} concentration and intracellular concentrations of cyclic AMP and diacylglycerol (Prentki & Matschinsky, 1987). The transduction of these signals is believed to occur through activation of Ca^{2+} /calmodulin-dependent protein kinase, cyclic AMP-dependent protein kinase and protein kinase C. Although it is widely recognized that glucose-stimulated insulin secretion is dependent on Ca^{2+} mobilization and that glucose-stimulated cyclic AMP accumulation potentiates Ca^{2+} -induced secretion, the role of protein kinase C in glucose-induced secretion is still debated. Glucose has been demonstrated to stimulate diacylglycerol formation in rat islets (Peter-Riesch *et al.*, 1988) and to induce protein kinase C-mediated protein phosphorylation in neonatal islets (Dunlop & Larkins, 1986). Furthermore, we have recently found evidence to suggest that glucose-induced cyclic AMP formation in mouse islets is mediated by protein kinase C (Thams *et al.*,

1988). However, other data have cast doubt on the significance of protein kinase C in glucose-induced secretion. Down-regulation of protein kinase C, which may be observed after prolonged exposure to TPA, was found to be without effect on glucose-induced secretion in rat islets (Hii *et al.*, 1987; Metz, 1988). Furthermore, several inhibitors of protein kinase C have been shown to be rather poor inhibitors of glucose-induced secretion. Thus, clomiphene was shown to cause a selective inhibition of TPA-induced insulin secretion (Hughes & Ashcroft, 1988), and H-7 was found to abolish TPA-induced and carbamoylcholine-induced insulin secretion while leaving glucose-induced secretion almost unaffected (Malaisse & Sener, 1985). However, these results were all obtained in static incubations, comprising both phases 1 and 2 of glucose-induced secretion.

In seeking an explanation for these discrepancies, the present study aimed to investigate the effects of down-regulation of protein kinase C on the dynamics of insulin secretion in mouse islets. The results substantiate a role for protein kinase C in glucose-induced insulin secretion and suggest that protein kinase C may inhibit phase 1 and stimulate phase 2 of glucose-induced secretion.

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; 4 α -PDD, 4 α -phorbol 12,13-didecanoate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine.

† To whom correspondence should be addressed.

EXPERIMENTAL

Materials

Crude bacterial collagenase and staurosporine were obtained from Boehringer, Mannheim, Germany. Tes, Hepes, EGTA, dithiothreitol, phosphatidylserine, 4 α -PDD, TPA, carbamoylcholine chloride, adrenaline and clonidine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Human serum albumin was from Behringwerke A.G., Marburg, Germany. [γ - 32 P]ATP (sp. radioactivity 3000 Ci/mmol) was from Amersham International, Amersham, Bucks., U.K. 125 I-insulin and guinea-pig anti-insulin serum were kindly provided by Nordisk Gentofte A/S, Gentofte, Denmark. All other chemicals were of analytical grade.

Preparation of islets

Islets were prepared by collagenase digestion of the pancreases of male albino mice (NMRI) (approx. 18–22 g body wt.) fed *ad libitum* on a standard laboratory diet. Islets were kept in tissue culture for 22–24 h in TCM 199 medium (1.26 mM- Ca^{2+} , 5.5 mM-glucose) or RPMI 1640 medium (0.43 mM- Ca^{2+} , 8.3 mM-glucose) supplemented with 10% (v/v) newborn-calf serum (Gibco), 20 mM-Hepes, 5 mM- NaHCO_3 , 100 units of penicillin/ml, 100 μg of streptomycin/ml and further additions where indicated.

Phosphorylation assay

Groups of 50 mouse islets were collected in 200 μl of 25 mM-Tes buffer, pH 6.90, containing 5 mM- MgCl_2 , 1 mM-EGTA, 0.1 mM-dithiothreitol, 0.01% (w/v) leupeptin and 10 units of aprotinin/ml. A homogenate was prepared by sonication (10 s, 40 W). For solubilization of membrane-bound protein kinase activity, the homogenate was incubated for 1 h at 4 °C in the above buffer supplemented with 0.125% Triton X-100. The solubilized preparation of homogenate was frozen in liquid N_2 and stored at –80 °C until the next day.

Samples of homogenate (corresponding to 7–10 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 and 0.2 mg of lysine-rich histone/ml, and further additions as indicated in the Figures. 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 serum albumin/ml as a carrier protein. The acid-precipitable radioactivity was then determined after three cycles of solubilization in 0.1 ml of 1 mM- NaOH and precipitation with 2 ml of 25% trichloroacetic acid.

Insulin release

Insulin release from islets was measured by perfusion in a non-circulating system with beads of polyacrylamide as a supporting medium, essentially as described by Knudsen *et al.* (1983). The present device allowed the simultaneous perfusion of six columns. In each experiment 25 islets were added on top of a column consisting of 0.25 ml of Bio-Gel P-2 (Bio-Rad Laboratories, Rockville Center, NY, U.S.A.) and carefully mixed with the beads. The islets were perfused at 37 °C at a flow rate of 0.26 ml/min. The perfusion medium was

Krebs–Ringer medium supplemented with 20 mM-Hepes, 5 mM- NaHCO_3 , 2 mg of human serum albumin/ml and 3.3 mM-glucose. Islets were perfused for 45 min with 3.3 mM-glucose to obtain a basal release rate and then challenged with different insulin secretagogues and test agents as indicated in the Figures and Tables. The effluent medium was collected for periods of 5 or 10 min and stored at –20 °C until assayed for insulin content. The rate of insulin release was expressed as ng of insulin/min per 100 islets.

Islet insulin content

Groups of 10 islets were collected in 1 ml of 0.7 M-HCl/ethanol (1:3, v/v) and incubated for approx. 20 h at 4 °C for extraction of insulin (Trimble & Renold, 1981).

Insulin assay

Insulin was determined by radioimmunoassay using a guinea-pig anti-insulin serum, monoiodinated pig insulin as tracer, rat insulin as standard, and ethanol to separate antibody-bound from free insulin.

Miscellaneous

4 α -PDD, TPA and staurosporine were added in a small volume of dimethyl sulphoxide, final concn. 0.01–0.1% (v/v).

Results are given as means $\pm$ S.E.M. for *n* experiments. Statistical evaluation of the data was made by Student's paired *t* test; NS, not significant ($P > 0.05$).

RESULTS

Effects of TPA pre-exposure on glucose-induced and TPA-induced insulin secretion

Fig. 1 shows the dynamics of insulin release from column-perfused mouse islets in response to glucose (16.7 mM) and TPA (0.16 μM). Islets were cultured for 22–24 h in TCM 199 culture medium containing 5.5 mM-glucose and 1.26 mM- Ca^{2+} (Fig. 1a) or in RPMI 1640 culture medium containing 8.3 mM-glucose and 0.43 mM- Ca^{2+} (Fig. 1b). In control islets from both media, stimulation by glucose (16.7 mM) was characterized by a dominant phase 2 of secretion and a smaller non-distinct phase 1 of secretion (45–55 min). Addition of TPA (0.16 μM) led to a transient potentiation of glucose (16.7 mM)-induced insulin secretion. At a non-stimulatory concentration of glucose (3.3 mM), TPA (0.16 μM) caused a slowly rising rate of secretion, comparable with phase 2 of glucose (16.7 mM)-induced secretion (Fig. 1a, inset).

A 22–24 h exposure in TCM 199 culture medium to TPA (0.16 μM) was associated with a complete loss of both glucose-induced and TPA-induced insulin secretion (Fig. 1a). In contrast, a 22–24 h exposure to TPA (0.16 μM) in RPMI 1640 culture medium preserved and potentiated phase 1 of glucose-induced secretion, while inhibiting phase 2 of glucose-induced secretion and abolishing TPA-induced secretion (Fig. 1b). Thus, although the total insulin released during 40 min of perfusion with glucose (16.7 mM) (45–85 min) was unaffected (Table 1), the percentage released during phase 1 (45–55 min) was increased from 12.9 ± 1.5 (4)% in controls to 35.8 ± 3.9 (4)% in TPA-treated islets ($P < 0.01$), and the percentage released during phase 2 (65–85 min) was decreased from 63.2 ± 3.9 (4)% to

Fig. 1. Effects of TPA pre-exposure on glucose-induced and TPA-induced insulin secretion

The dynamics of insulin release from column-perfused islets in response to 3.3 mM-glucose, 16.7 mM-glucose and 16.7 mM-glucose + 0.16 μ M-TPA were determined as described in the Experimental section. Islets were pretreated with (○) or without (●) 0.16 μ M-TPA for 22–24 h in (a) TCM 199 (5.5 mM-glucose, 1.26 mM- Ca^{2+}) or (b) RPMI 1640 (8.3 mM-glucose, 0.43 mM- Ca^{2+}) culture medium. The inset in (a) shows the effect of 0.16 μ M-TPA at 3.3 mM-glucose on insulin release from control islets cultured in TCM 199 medium. Results are means $\pm$ S.E.M. ($n = 4-6$).

35.3 ± 1.4 (4)% ($P < 0.005$) (Table 2). Inclusion of the inactive phorbol ester 4 α -PDD (0.16 μ M) in either RPMI 1640 or TCM 199 medium did not affect glucose-induced or TPA-induced insulin secretion (results not shown).

Inclusion of adrenaline (10 μ M) in TCM 199 medium during TPA exposure preserved an ability for glucose to stimulate secretion in subsequent perfusion experiments (Fig. 2a, Table 1). Thus glucose (16.7 mM) stimulation of islets pretreated in adrenaline (10 μ M) + TPA (0.16 μ M)-supplemented medium exhibited a potentiated phase 1 of secretion (45–55 min), followed by a sustained, non-rising, phase 2 of secretion (65–85 min) (Fig. 2a, Table 2). Inclusion of adrenaline (10 μ M) itself did not affect glucose-induced secretion (Tables 1 and 2). Furthermore, adrenaline (10 μ M) did not prevent down-regulation of TPA-inducible secretion during TPA exposure in TCM 199 medium (Fig. 2a). Similar results were obtained with clonidine (10 μ M), a specific α_2 -adrenergic agonist (Fig. 2b). Thus, although the total insulin release during 45–85 min of perfusion with glucose (16.7 mM) was unaffected after exposure to TPA in the presence of either adrenaline or clonidine (Table 1), down-regulation caused a shift in the time course of glucose-induced secretion (Table 2).

Effect of TPA pre-exposure on carbamoylcholine-induced insulin secretion

Fig. 3(a) shows the time course of carbamoylcholine-induced insulin secretion from islets after culture in TCM

199 medium. At 3.3 mM-glucose, carbamoylcholine (100 μ M) elicited a biphasic response, consisting of a dominant phase 1 (45–55 min) and a small phase 2 (65–85 min) of secretion. At 16.7 mM-glucose, carbamoylcholine (100 μ M) caused a persistent potentiation of glucose-induced secretion (185–235 min) (Fig. 3a). All responses to carbamoylcholine were abolished in TPA-pretreated islets (Fig. 3a). Inclusion of adrenaline (10 μ M) in TCM 199 during TPA exposure, however, retained and potentiated the ability of carbamoylcholine at 3.3 mM-glucose to induce phase 1 of secretion (Fig. 3b). Thus, although the total insulin released during 40 min of perfusion with carbamoylcholine (100 μ M) (45–85 min) was unaffected (Table 3), the percentage released during phase 1 (45–55 min) was increased from 36.2 ± 4.1 (4)% in adrenaline-pretreated islets to 78.2 ± 8.2 (4)% in adrenaline + TPA-pretreated islets ($P < 0.005$), and the percentage released during phase 2 (65–85 min) was decreased from 44.9 ± 4.0 (4)% to 11.8 ± 4.0 (4)% ($P < 0.005$) (Table 4). Adrenaline (10 μ M) itself did not affect total insulin release (Table 3), but tended to show a slight effect on the time course, increasing the fractional output during phase 2 of secretion (Table 4).

In line with an inhibitory effect of down-regulation of protein kinase C on phase 2 of carbamoylcholine-induced secretion at 3.3 mM-glucose, pretreatment with adrenaline + TPA abolished the potentiating effect of carbamoylcholine at 16.7 mM-glucose (185–235 min) (Fig. 3b, Table 3).

Table 1. Effect of TPA pretreatment on glucose-induced insulin secretion

Islets were pretreated for 22–24 h in TCM 199 (1.26 mM-Ca²⁺, 5.5 mM-glucose) or RPMI 1640 (0.43 mM-Ca²⁺, 8.3 mM-glucose) culture medium and with further additions as indicated. Perfusion of islets was performed as described in the corresponding Figs. 1, 2, 5 and 6. Results are means $\pm$ S.E.M. for the numbers of experiments in parentheses, and are given as the mean rates of insulin release at 3.3 mM-glucose (15–45 min) and 16.7 mM-glucose (45–85 min). The statistical differences from paired control values were determined by a paired *t* test: NS, not significant (*P* > 0.05).

Culture		Perfusion	Insulin release (ng/min per 100 islets)		P	
Line	Medium	Added agents	Added agent	Glucose (3.3 mM) (15–45 min)		Glucose (16.7 mM) (45–85 min)
1	TCM 199	–		0.83 ± 0.35 (15)	12.42 ± 1.46 (15)	Versus 1: < 0.001 Versus 1: NS Versus 3: NS
2		TPA (0.16 μM)		0.41 ± 0.14 (12)	1.66 ± 0.44 (12)	
3		Adrenaline (10 μM)		2.78 ± 1.29 (5)	10.52 ± 1.62 (5)	
4		Adrenaline (10 μM) + TPA (0.16 μM)		2.07 ± 0.60 (5)	20.20 ± 5.16 (5)	
5		Clonidine (10 μM)		0.33 ± 0.17 (3)	21.75 ± 9.65 (3)	Versus 1: NS Versus 5: NS
6		Clonidine (10 μM) + TPA (0.16 μM)		1.74 ± 0.07 (3)	21.97 ± 5.17 (3)	
7		EGTA (1 mM)		2.92 ± 0.61 (3)	24.71 ± 5.24 (3)	Versus 1: NS Versus 7: NS
8		EGTA (1 mM) + TPA (0.16 μM)		1.84 ± 0.94 (3)	20.37 ± 3.31 (3)	
9		–	Staurosporine (1 μM)	1.17 ± 0.62 (2)	15.85 ± 5.57 (3)	Versus 1: NS Versus 10: NS
10	RPMI 1640	–		0.38 ± 0.26 (4)	8.67 ± 4.78 (4)	
11		TPA (0.16 μM)		1.43 ± 0.67 (4)	6.80 ± 1.98 (4)	

Table 2. Effect of TPA pretreatment on the time course of glucose-induced insulin secretion

Islets were pretreated for 22–24 h in TCM 199 or RPMI 1640 culture medium and with further additions as indicated. Perfusion of islets was performed as described in the corresponding Figs. 1, 2, 5 and 6. Total insulin release during phase 1 (45–55 min), phase 2 (65–85 min) and the whole period (45–85 min) of stimulation with 16.7 mM-glucose was calculated. The results are means $\pm$ S.E.M. for the numbers of experiments in parentheses, and are given as the percentages released during phases 1 (45–55 min) and 2 (65–85 min). The statistical differences from paired control values were determined by a paired *t* test: NS, not significant (*P* > 0.05). ND, not determined.

Line	Culture		Insulin release [% of total (45–85 min)]		
	Medium	Added agents	Perfusion	Phase 1 (45–55 min)	Phase 2 (65–85 min)
			Added agent	<i>P</i>	<i>P</i>
1	TCM 199	–		15.56 $\pm$ 0.75 (15)	62.52 $\pm$ 1.37 (15)
2		TPA (0.16 μ M)		ND	ND
3		Adrenaline (10 μ M)		16.89 $\pm$ 1.92 (5)	59.28 $\pm$ 2.01 (5)
4		Adrenaline (10 μ M) + TPA (0.16 μ M)		29.38 $\pm$ 1.15 (5)	46.09 $\pm$ 1.24 (5)
5		Clonidine (10 μ M)		10.04 $\pm$ 2.81 (3)	65.53 $\pm$ 2.00 (3)
6		Clonidine (10 μ M) + TPA (0.16 μ M)		29.68 $\pm$ 3.95 (3)	42.39 $\pm$ 0.37 (3)
7		EGTA (1 mM)		20.94 $\pm$ 2.35 (3)	56.52 $\pm$ 1.73 (3)
8		EGTA (1 mM) + TPA (0.16 μ M)		37.01 $\pm$ 3.42 (3)	39.69 $\pm$ 4.27 (3)
9		–	Staurosporine (1 μ M)	35.71 $\pm$ 4.82 (3)	34.36 $\pm$ 6.00 (3)
10	RPMI 1640	–		12.85 $\pm$ 1.54 (4)	63.19 $\pm$ 3.92 (4)
11		TPA (0.16 μ M)		35.82 $\pm$ 3.93 (4)	35.32 $\pm$ 1.39 (4)
				Versus 10: < 0.01	Versus 10: < 0.005
				Versus 1: NS	Versus 1: NS
				Versus 3: < 0.001	Versus 3: < 0.005
				Versus 5: < 0.001	Versus 5: < 0.001
				Versus 7: < 0.005	Versus 7: < 0.05
				Versus 1: < 0.02	Versus 1: < 0.05

Table 3. Effect of TPA pretreatment on carbamoylcholine-induced insulin secretion

Islets were pretreated for 22–24 h in TCM 199 culture medium and with further additions as indicated. Perfusion of islets was performed as described in the corresponding Fig. 3. Results are means $\pm$ S.E.M. for the numbers of experiments in parentheses, and are given as the mean rates of insulin release at 3.3 mM-glucose + 100 μ M-carbamoylcholine (45–85 min), 16.7 mM-glucose (165–185 min) and 16.7 mM-glucose + 100 μ M-carbamoylcholine (185–235 min). The statistical differences from paired control values were determined by a paired *t* test: NS, not significant (*P* > 0.05).

Line	Culture		Insulin release (ng/min per 100 islets)		
	Medium	Added agents	Perfusion	Glucose (16.7 mM) + carbamoylcholine (100 μ M) (185–235 min)	<i>P</i> (versus 16.7 mM-glucose)
			Glucose (3.3 mM) + carbamoyl- choline (100 μ M) (45–85 min)	Glucose (16.7 mM) (165–185 min)	
				<i>P</i>	
1	TCM 199	–		9.65 $\pm$ 2.51 (3)	26.84 $\pm$ 3.44 (3)
2		TPA (0.16 μ M)		4.13 $\pm$ 1.04 (3)	6.09 $\pm$ 1.04 (3)
3		Adrenaline (10 μ M)		8.56 $\pm$ 0.89 (4)	22.61 $\pm$ 3.04 (4)
4		Adrenaline (10 μ M) + TPA (0.16 μ M)		19.72 $\pm$ 4.79 (4)	19.67 $\pm$ 5.09 (4)
				Versus 1: < 0.01	< 0.005
				Versus 1: NS	NS
				Versus 3: NS	< 0.005
					NS

Fig. 2. Effect of adrenaline and clonidine on TPA-induced down-regulation of glucose-induced insulin secretion

Insulin release from column-perfused mouse islets in response to 3.3 mM-glucose, 16.7 mM-glucose and 16.7 mM-glucose + 0.16 μ M-TPA was determined as described in the Experimental section. Islets were pretreated for 22–24 h with (○) or without (●) 0.16 μ M-TPA in (a) TCM 199 medium supplemented with adrenaline (10 μ M) or (b) TCM 199 medium supplemented with clonidine (10 μ M). Results are means $\pm$ S.E.M. ($n = 3$ –5).

Fig. 3. Effect of TPA pre-exposure on carbamoylcholine-induced insulin secretion

Insulin release from islets in response to 3.3 mM-glucose, 3.3 mM-glucose + 100 μ M-carbamoylcholine, 16.7 mM-glucose and 16.7 mM-glucose + 100 μ M-carbamoylcholine was determined as described in the Experimental section. Islets were pretreated for 22–24 h with (○) or without (●) 0.16 μ M-TPA in (a) TCM 199 medium or (b) TCM 199 medium supplemented with 10 μ M-adrenaline. Results are means $\pm$ S.E.M. ($n = 3$ –4).

Effect of TPA on down-regulation of protein kinase C

A 22–24 h exposure in TCM 199 culture medium to TPA (0.16 μ M) decreased total islet protein kinase C to approx. 8% of control values (Fig. 4). In comparison, exposure to the inactive phorbol ester 4 α -PDD did not affect protein kinase C activity. Complete down-regulation of protein kinase C in response to TPA was not achieved. Prolonged exposure for 48 h to TPA (0.16 μ M), which was a maximum stimulating concentration, appeared to cause a total abolition of activity, but was associated with a cell loss of approx. 50% (results not shown).

Neither adrenaline (10 μ M) nor clonidine (10 μ M) influenced protein kinase C itself or affected TPA-induced down-regulation of protein kinase C (Fig. 4). Thus total protein kinase C in homogenate amounted to 0.37, 0.42 and 0.01 pmol/2 min per 50 islets in islets treated with TPA, TPA + adrenaline and TPA + clonidine respectively.

Effect of TPA on islet insulin content

The continuous exposure of islets to TPA during down-regulation of protein kinase C caused a decrease in islet insulin content (Table 5). As compared with control islets cultured in the absence of phorbol ester, a 22–24 h exposure to TPA decreased insulin from 36 to 15 ng/islet and from 55 to 26 ng/islet in TCM 199 and RPMI 1640 media respectively. Addition of adrenaline (10 μ M) or clonidine (10 μ M) to TCM 199 medium did not affect insulin content and did not prevent a major TPA-induced loss in insulin content (Table 5). In addition, inclusion of EGTA (1 mM) in TCM 199 showed no effect on insulin content in the absence or presence of TPA (Table 5).

Table 4. Effect of TPA pretreatment on the time course of carbamoylcholine-induced insulin secretion

Islets were pretreated for 22–24 h in TCM 199 culture medium and with further additions as indicated. Perfusion of islets was performed as described in the corresponding Fig. 3. Total insulin release during phase 1 (45–55 min), phase 2 (65–85 min) and the whole period (45–85 min) of stimulation with 100 μ M-carbamoylcholine at 3.3 mM-glucose was calculated. The results are means $\pm$ S.E.M. for the numbers of experiments in parentheses, and are given as the percentages released during phases 1 (45–55 min) and 2 (65–85 min). The statistical differences from paired control values were determined by a paired *t* test: NS, not significant ($P > 0.05$). ND, not determined.

Line	Culture		Insulin release [% of total (45–85 min)]			
	Medium	Added agents	Phase 1 (45–55 min)	<i>P</i>	Phase 2 (65–85 min)	<i>P</i>
1	TCM 199	–	45.43 $\pm$ 3.65 (4)		39.70 $\pm$ 4.05 (3)	
2		TPA (0.16 μ M)	ND		ND	
3		Adrenaline (10 μ M)	36.23 $\pm$ 4.12 (4)	Versus 1: NS	44.86 $\pm$ 3.97 (4)	Versus 1: < 0.05
4		Adrenaline (10 μ M) + TPA (0.16 μ M)	78.15 $\pm$ 8.16 (4)	Versus 3: < 0.005	11.80 $\pm$ 4.01 (4)	Versus 3: < 0.005

Effect of Ca^{2+} in TCM 199 medium on insulin secretion

The ability of α_2 -adrenergic agonists to preserve and potentiate phase 1 of glucose-induced secretion in TPA-exposed islets (Fig. 2) may rely on an inhibitory effect of these agents on Ca^{2+} uptake (Wollheim *et al.*, 1977) or on distal steps in Ca^{2+} -induced secretion (Tamagawa *et al.*, 1985; Jones *et al.*, 1987) during down-regulation in TCM 199 medium. Furthermore, the observed difference

Fig. 4. Down-regulation of protein kinase C

Protein kinase C activity was assessed after pretreatment of islets in TCM 199 culture medium for 22–24 h with the indicated concentrations of 4α-PDD, TPA, adrenaline and clonidine. Protein kinase C activity in islet homogenate was determined as described in the Experimental section. The activity in the presence of Ca^{2+} (100 μ M) + TPA (32 nM) + phosphatidylserine (20 μ g/ml) corrected for the activity in the sole presence of Ca^{2+} (100 μ M) is given. Phosphatidylserine 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, and 1.1 mM- CaCl_2 was added to obtain a free Ca^{2+} concentration of 100 μ M. Results are means $\pm$ S.E.M. ($n = 5$). The statistical significance of the differences from control islets pretreated in the absence of TPA was assessed by using a paired *t* test: * $P < 0.02$; ** $P < 0.001$.

between TCM 199 and RPMI 1640 in preserving phase 1 of glucose-induced secretion (Fig. 1) may be due to the lower Ca^{2+} concentration in RPMI 1640 (0.43 mM) relative to TCM 199 (1.26 mM). Therefore, to investigate whether Ca^{2+} may have an abolishing effect on glucose-induced secretion during down-regulation of protein kinase C in TCM 199, the effect of the Ca^{2+} -chelating agent EGTA (1 mM) was examined.

A decrease in Ca^{2+} from 1.26 mM to 0.26 mM in TCM 199 did not itself have any influence on glucose-induced insulin secretion (Tables 1 and 2). In line with the above considerations, however, EGTA (1 mM) in combination with TPA led to preservation and potentiation of phase 1 (45–55 min) of glucose-induced insulin secretion, but decreased phase 2 (65–85 min) of glucose-induced secretion and abolished TPA-induced (105–165 min) secretion (Fig. 5). Thus, although total insulin release with glucose (16.7 mM) (45–85 min) was unchanged (Table 1), the fractional release during phase 1 (45–55 min) was enhanced from 20.9 $\pm$ 2.4 (3)% in EGTA-exposed islets to 37.0 $\pm$ 3.4 (3)% in EGTA+TPA-exposed islets ($P < 0.005$), and the fractional release during phase 2 was decreased from 56.5 $\pm$ 1.7 (3)% to 39.7 $\pm$ 4.3 (3)% (Table 2).

In addition to Ca^{2+} , the concentration of glucose in the media was critical. An increase in glucose from 8.3 mM to the conventional 11 mM in RPMI 1640 during TPA exposure led to abolition of both phases of glucose-induced secretion in subsequent perfusion experiments (results not shown).

Effect of staurosporine on insulin secretion

To substantiate further a role for protein kinase C in glucose-induced insulin secretion, the effect of staurosporine, an inhibitor of protein kinase C (Tamaoki *et al.*, 1986), was investigated. Staurosporine (1 μ M) caused total abolition of TPA-stimulated release (105–165 min) (Fig. 6). This inhibition of TPA-induced secretion was associated with a potentiation of phase 1 and a decrease in phase 2 of glucose-induced secretion. Thus the percentage released during phase 1 (45–55 min) was increased from 14.0 $\pm$ 1.6 (3)% in controls to 35.7 $\pm$ 4.8 (3)% in staurosporine-perfused islets ($P < 0.02$), and the percentage released during phase 2 was decreased from 61.0 $\pm$ 3.6 (3)% to 34.4 $\pm$ 6.0 (3)% ($P < 0.05$) (Table 2).

Table 5. Effect of culture conditions on insulin content in mouse islets

Islet insulin content was measured after culture for 22–24 h in TCM 199 or RPMI 1640 culture medium and with additions as indicated. Results are means $\pm$ S.E.M. for the numbers of experiments given in parentheses. The statistical differences from paired control values were determined by a paired *t* test: NS, not significant ($P > 0.05$).

Line	Culture		Insulin content	
	Medium	Added agents	(ng/islet)	<i>P</i>
1	TCM 199	–	36.1 $\pm$ 5.7 (11)	
2		TPA (0.16 μ M)	14.5 $\pm$ 2.6 (11)	Versus 1: < 0.001
3		Adrenaline (10 μ M)	39.8 $\pm$ 8.2 (8)	Versus 1: NS
4		Adrenaline (10 μ M) + TPA (0.16 μ M)	22.9 $\pm$ 5.2 (8)	Versus 3: < 0.02; Versus 2: < 0.02
5		Clonidine (10 μ M)	37.8 $\pm$ 6.0 (3)	Versus 1: NS
6		Clonidine (10 μ M) + TPA (0.16 μ M)	22.7 $\pm$ 1.6 (3)	Versus 5: < 0.05; Versus 2: NS
7		EGTA (1 mM)	33.3 $\pm$ 3.5 (3)	Versus 1: NS
8		EGTA (1 mM) + TPA (0.16 μ M)	13.1 $\pm$ 1.6 (3)	Versus 7: < 0.02; Versus 2: NS
9	RPMI 1640	–	55.1 $\pm$ 14.9 (4)	
10		TPA (0.16 μ M)	26.2 $\pm$ 7.7 (4)	Versus 9: < 0.02

Staurosporine (1 μ M) was without effect on both basal release at 3.3 mM-glucose (15–45 min) and the total amount of insulin released during 40 min of perfusion with 16.7 mM-glucose (45–85 min) (Table 1).

Comparative studies with rat islets

In previous studies with rat islets, an abolishing effect of TPA pretreatment on glucose-induced insulin secretion was not observed (Hii *et al.*, 1987; Metz, 1988). In view of this apparent discrepancy with our results with mouse islets, comparative experiments with rat islets were per-

formed. In our hands, rat and mouse islets show identical responses, and, as observed with mouse islets (Fig. 1a), TPA pretreatment of rat islets in normal TCM 199 medium leads to an abolition of both phases 1 and 2 of glucose-induced insulin secretion (results not shown). Thus no obvious species differences are involved. In view of the profound effect of Ca^{2+} lowering in TCM 199 (Figs. 1a and 5), it seems likely that the observed deviations may rely on differences in the Ca^{2+} -sensitivity of the islet preparations used or differences in the Ca^{2+} concentration of the media used for TPA pretreatment.

Fig. 5. Role of Ca^{2+} in TCM 199 medium on TPA-induced abolition of glucose-induced insulin secretion

Insulin release from column-perfused islets was determined as described in Fig. 1. Islets were pretreated with (○) or without (●) 0.16 μ M-TPA for 22–24 h in TCM 199 medium supplemented with 1 mM-EGTA. Results are means $\pm$ S.E.M. ($n = 3$).

Fig. 6. Effects of staurosporine on glucose-induced and TPA-induced insulin secretion

The dynamics of insulin release from islets maintained in TCM 199 medium for 22–24 h were determined as described in Fig. 1. Islets were perfused in the absence (●) or presence (○) of 1 μ M-staurosporine and further additions as indicated. Results are means $\pm$ S.E.M. ($n = 3$).

DISCUSSION

The results substantiate a role for protein kinase C in glucose-induced insulin secretion and suggest that protein kinase C may inhibit phase 1 and stimulate phase 2 of glucose-induced insulin secretion.

Previous observations with rat and mouse islets have demonstrated that TPA in the absence of stimulatory concentrations of glucose causes a slowly progressive increase in insulin secretion (Malaisse *et al.*, 1980; Zawulich *et al.*, 1983; Henquin *et al.*, 1987). A similar slow increase in insulin secretion in response to TPA was also observed in the present study. On the basis of this slow response to TPA, it has previously been suggested that protein kinase C is mainly involved in phase 2 of glucose-induced secretion (Zawulich *et al.*, 1983). In support of this hypothesis, early studies demonstrated that the non-specific protein kinase C inhibitor vitamin A (Taffet *et al.*, 1983) showed a preferential inhibition of phase 2 of glucose-induced secretion (Chertow *et al.*, 1979). The present results substantiate a stimulatory role for protein kinase C in phase 2 of glucose-induced secretion. Thus both down-regulation of protein kinase C in RPMI 1640 or in TCM 199 supplemented with α_2 -adrenergic agonists or EGTA and direct inhibition of protein kinase C with staurosporine resulted in an inhibition of the progressively increasing phase 2 of glucose-induced insulin secretion.

It is accepted that glucose has the capacity to stimulate both phosphoinositide hydrolysis and diacylglycerol accumulation in islets (Best, 1986; Zawulich & Zawulich, 1988; Peter-Riesch *et al.*, 1988), and it has been demonstrated that glucose-induced phosphoinositide hydrolysis may parallel that biphasic pattern of insulin secretion (Zawulich & Zawulich, 1988). Furthermore, several studies have shown that glucose in islets stimulates the release of arachidonic acid (Wolf *et al.*, 1986), a putative activator of several protein kinase C isoenzymes (Nishizuka, 1988). However, recent observations in rat islets have cast doubt on the involvement of protein kinase C in glucose-induced secretion. It has been reported that down-regulation of protein kinase C does not affect glucose-induced insulin secretion (Hii *et al.*, 1987; Metz, 1988). Furthermore, the protein kinase C inhibitors clomiphene and H-7 did not inhibit glucose-induced insulin secretion, but abolished TPA-induced secretion (Malaisse & Sener, 1985; Hughes & Ashcroft, 1988; Metz, 1988). However, these results were obtained in static incubations, comprising both phases 1 and 2 of glucose-induced secretion. According to the present results, a stimulatory action of protein kinase C during phase 2 is counteracted by a major inhibitory effect of protein kinase C during phase 1 of glucose-induced insulin secretion, thus offering an explanation for the previous observed discrepancies.

At first glance, a concomitant inhibitory and stimulatory effect of protein kinase C may seem contradictory. However, previous studies have suggested that protein kinase C may have roles in the negative-feedback regulation of insulin secretion. Thus TPA has been found to attenuate vasopressin-stimulated phosphoinositide hydrolysis in RIN insulinoma cells (Monaco *et al.*, 1988). In line with similar observations in other tissues, this effect would decrease both InsP_3 -induced Ca^{2+} mobilization from the endoplasmic reticulum and diacylglycerol activation of protein kinase C (Berridge, 1987).

The exact locus for this feedback inhibition is not known and may involve receptors, G-proteins and phospholipase C. However, recent studies in mouse islets suggest that protein kinase C activity has the capacity to inhibit phosphoinositide-specific phospholipase C directly (Hedekov *et al.*, 1988), suggesting that this effect of protein kinase C may comprise glucose-induced phosphoinositide hydrolysis as well. Furthermore, protein kinase C has been shown to stimulate the InsP_3 5'-phosphomonoesterase in tissues (Connolly *et al.*, 1986), an effect which also would attenuate InsP_3 -induced Ca^{2+} mobilization. Other evidence suggest that protein kinase C may activate the plasma-membrane-associated $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase and stimulate Ca^{2+} efflux from cells (Smallwood *et al.*, 1988). Accordingly, TPA has been found to decrease the K^+ -induced increase in cytosolic Ca^{2+} in the clonal insulin-secreting RINm5F cell line (Arkhammar *et al.*, 1986) and to stimulate Ca^{2+} efflux from rat islets (Malaisse *et al.*, 1980). It is tempting to speculate that the efficacy of protein kinase C depletion to potentiate phase 1 of glucose-induced and carbamoylcholine-induced insulin secretion in part may reflect an abolition of these regulatory effects of protein kinase C on both phosphoinositide hydrolysis and Ca^{2+} fluxes.

The ability of both adrenaline and clonidine to preserve phase 1 of glucose-induced secretion during down-regulation of protein kinase C suggests that this effect is exerted through α_2 -adrenergic receptors. It is known that α_2 -adrenergic stimulation inhibits both glucose-induced and TPA-induced insulin secretion (Schuit & Pipeleers, 1986; Malaisse *et al.*, 1980). Surprisingly, however, neither adrenaline nor clonidine prevented a major loss in islet insulin content during TPA stimulation and down-regulation of protein kinase C (Table 5). Although it is not believed that α_2 -adrenergic agonists inhibit phosphoinositide hydrolysis (Montague *et al.*, 1985), they have been shown to inhibit glucose-induced Ca^{2+} influx (Wollheim *et al.*, 1977), cyclic AMP accumulation (Yamazaki *et al.*, 1982) and glucose oxidation (Laychock, 1987) in islets. In addition, a direct inhibitory effect on Ca^{2+} -induced exocytosis has been demonstrated in permeabilized islets (Tamagawa *et al.*, 1985; Jones *et al.*, 1987) and insulinoma cells (Ullrich & Wollheim, 1988). The mimicking effect of Ca^{2+} lowering in TCM 199 during down-regulation of protein kinase C points to an effect of adrenaline and clonidine on Ca^{2+} handling and Ca^{2+} -induced exocytosis in preserving phase 1 of glucose-induced secretion.

Previous studies with rat islets have shown that the Ca^{2+} -sensitivity of different islet preparations may vary to a large extent. Thus, in a study by Hughes *et al.* (1987) the concentration of extracellular Ca^{2+} required for half-maximal stimulation of insulin secretion by glucose was 60 μM , whereas in a study by Hii *et al.* (1986) the corresponding value exceeded 2 mM- Ca^{2+} . In comparison, we have found a value of approx. 0.8 mM- Ca^{2+} for half-maximal stimulation of insulin secretion by glucose and a threshold value for stimulation of approx. 0.2 mM- Ca^{2+} in 22–24 h-cultured mouse islets (results not shown). These values are in agreement with the observed sensitivity to Ca^{2+} during TPA pretreatment (Figs. 1a and 5). In our view, therefore, the ability observed herein, of TPA pretreatment to abolish glucose-induced secretion may have been by-passed in the previous studies (Hii *et al.*, 1987; Metz, 1988), owing to differences in the Ca^{2+} -sensitivity between different islet preparations. In ad-

dition, the deviations may be explained by differences in the composition of the media used for TPA pretreatment, but the Ca^{2+} concentrations in the media used in the previous studies were not specified.

It has previously been demonstrated that carbamoylcholine stimulates phosphoinositide hydrolysis in islets (Best, 1986; Morgan *et al.*, 1987). In accordance, the present results substantiate a role for protein kinase C in carbamoylcholine-induced insulin secretion. Although carbamoylcholine does not stimulate insulin secretion in the absence of glucose (Trus *et al.*, 1979), carbamoylcholine at 3.3 mM-glucose was found to induce a biphasic type of secretion (Fig. 3). In support of a stimulatory effect of protein kinase C during phase 2 of secretion, down-regulation of protein kinase C was found to abolish phase 2 of carbamoylcholine-induced secretion at 3.3 mM-glucose. Furthermore, down-regulation abolished the potentiating effect of carbamoylcholine observable on addition during phase 2 of glucose (16.7 mM)-induced secretion. Finally, in line with the observations for glucose, protein kinase C also appears to have negative modulatory roles in phase 1 of carbamoylcholine-induced secretion, altogether suggestive of a common mechanism for these two secretagogues in stimulation of insulin secretion.

The mechanism by which protein kinase C potentiates phase 2 of glucose-induced and carbamoylcholine-induced insulin secretion is not known. We have recently suggested that protein kinase C may have a role in glucose and carbamoylcholine stimulation of cyclic AMP accumulation in islets (Thams *et al.*, 1988), and most likely the potentiation of phase 2 of secretion involves an increased cyclic AMP accumulation. In contrast with TPA, however, cyclic AMP does not induce insulin secretion at non-stimulatory concentrations of glucose (Henquin, 1985). It is possible that the potentiating effect of protein kinase C on insulin secretion may involve several different effects.

In conclusion, the present results have clearly demonstrated that protein kinase C inhibits phase 1 and stimulates phase 2 of glucose-induced insulin secretion. Both down-regulation of protein kinase C and direct inhibition of the kinase were found to potentiate phase 1 and inhibit phase 2 of glucose-induced secretion. The implication of protein kinase C in glucose-induced secretion is strengthened by similar effects of protein kinase C depletion on the two phases of carbamoylcholine-induced secretion, which are thought to involve protein kinase C activation. The mechanism of glucose in protein kinase C activation, as well as the mechanisms for protein kinase C in regulation of phases 1 and 2 of secretion, remain to be resolved. It is suggested that the regulation by protein kinase C comprises both an attenuating effect on phosphoinositide hydrolysis and Ca^{2+} mobilization and an amplifying effect on cyclic AMP accumulation in response to glucose.

This work was supported by financial aid from the Danish Medical Research Council (grant no. 12-8195), NOVO's Foundation, Nordic Insulin Foundation and the Danish Diabetes Association. The skilful technical assistance of Mr. Peter V. Larsen is highly appreciated.

REFERENCES

- Arkhammar, P., Nilsson, T. & Berggren, P.-O. (1986) *Biochim. Biophys. Acta* **887**, 236–241
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Best, L. (1986) *Biochem. J.* **238**, 773–779
- Chertow, B. S., Buschmann, R. J. & Kaplan, R. (1979) *Diabetes* **28**, 754–761
- Connolly, T. M., Lawing, W. J. & Majerus, P. W. (1986) *Cell* **46**, 951–958
- Dunlop, M. E. & Larkins, R. G. (1986) *Arch. Biochem. Biophys.* **248**, 562–569
- Hedekov, C. J., Capito, K., Gembal, M., Malik, T. & Thams, P. (1988) *Diabetes Res. Clin. Pract.* **5** (suppl. 1), S14
- Henquin, J. C. (1985) *Arch. Int. Physiol. Biochim.* **93**, 37–48
- Henquin, J. C., Bozem, M., Schmeer, W. & Nenquin, M. (1987) *Biochem. J.* **246**, 393–399
- Hii, C. S. T., Stutchfield, J. & Howell, S. L. (1986) *Biochem. J.* **233**, 287–289
- Hii, C. S. T., Jones, P. M., Persaud, S. J. & Howell, S. L. (1987) *Biochem. J.* **246**, 489–493
- Hughes, S. J. & Ashcroft, S. J. H. (1988) *Biochem. J.* **249**, 825–830
- Hughes, S. J., Christie, M. R. & Ashcroft, S. J. H. (1987) *Mol. Cell. Endocrinol.* **50**, 231–236
- Jones, P. M., Fyles, J. M., Persaud, S. J. & Howell, S. L. (1987) *FEBS Lett.* **219**, 139–144
- Knudsen, P., Kofod, H., Lernmark, Å. & Hedekov, C. J. (1983) *Am. J. Physiol.* **245**, E338–E346
- Laychock, S. G. (1987) *Mol. Pharmacol.* **32**, 241–248
- Malaisse, W. J. & Sener, A. (1985) *IRCS Med. Sci.* **13**, 1239–1240
- Malaisse, W. J., Sener, A., Herchuelz, A., Carpinelli, A. R., Poloczek, P., Winand, J. & Castagna, M. (1980) *Cancer Res.* **40**, 3827–3831
- Metz, S. A. (1988) *Diabetes* **37**, 3–7
- Monaco, M. E., Levy, B. L. & Richardson, S. B. (1988) *Biochem. Biophys. Res. Commun.* **151**, 717–724
- Montague, W., Morgan, N. G., Rumford, G. M. & Prince, C. A. (1985) *Biochem. J.* **227**, 483–489
- Morgan, N. G., Rumford, G. M. & Montague, W. (1987) *Biochem. J.* **244**, 669–674
- Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
- Peter-Riesch, B., Fathi, M., Schlegel, W. & Wollheim, C. B. (1988) *J. Clin. Invest.* **81**, 1154–1161
- Prentki, M. & Matschinsky, F. M. (1987) *Physiol. Rev.* **67**, 1185–1248
- Schuit, F. C. & Pipeleers, D. G. (1986) *Science* **232**, 875–877
- Smallwood, J. I., Gügi, B. & Rasmussen, H. (1988) *J. Biol. Chem.* **263**, 2195–2202
- Taffet, S. M., Greenfield, A. R. & Haddox, M. K. (1983) *Biochem. Biophys. Res. Commun.* **114**, 1194–1199
- Tamagawa, T., Niki, I., Niki, H. & Niki, A. (1985) *Biomed. Res.* **6**, 429–432
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Thams, P., Capito, K. & Hedekov, C. J. (1988) *Biochem. J.* **253**, 229–234
- Trimble, E. R. & Renold, A. E. (1981) *Am. J. Physiol.* **240**, E422–E427
- Trus, M. D., Hintz, C. S., Weinstein, J. B., Williams, A. D., Pagliara, A. S. & Matschinsky, F. M. (1979) *J. Biol. Chem.* **254**, 3921–3929
- Ullrich, S. & Wollheim, C. B. (1988) *J. Biol. Chem.* **263**, 8615–8620
- Wolf, B. A., Turk, J., Sherman, W. R. & McDonald, M. L. (1986) *J. Biol. Chem.* **261**, 3501–3511

Wollheim, C. B., Kikuchi, M., Renold, A. E. & Sharp, G. W. G. (1977) *J. Clin. Invest.* **60**, 1165–1173
Yamazaki, S., Katada, T. & Ui, M. (1982) *Mol. Pharmacol.* **21**, 648–653

Zawalich, W. S. & Zawalich, K. C. (1988) *Diabetes* **37**, 1294–1300
Zawalich, W., Brown, C. & Rasmussen, H. (1983) *Biochem. Biophys. Res. Commun.* **117**, 448–455

Received 12 June 1989/13 September 1989; accepted 11 October 1989