Effects of the phorbol ester phorbol 12-myristate 13-acetate (PMA) on islet-cell responsiveness

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Collagenase-isolated rat islets were labelled for 2 h in *myo*-[2-³H]inositol solution supplemented with 2.75 mM-glucose. The phorbol ester phorbol 12-myristate 13-acetate (PMA; 0.1 or 1 μ M) was also present in some experiments. After labelling, islets were washed and then perfused in 2.75 mM-glucose to establish basal [³H]inositol-efflux and insulin-secretory rates. Subsequently, the responses of these islets to stimulation with various agonists were assessed. Inositol phosphate accumulation was measured at the termination of the perfusion. In separate experiments, the cellular location of protein kinase C (PKC) after PMA pretreatment was measured by quantitative immunoblotting of membrane and cytosolic fractions. The following observations were made. (1) Labelling in 0.1–1 μ M-PMA had no deleterious effect on total [³H]inositol incorporation during the 2 h labelling period. However, islets labelled for 2 h in 1 μ M-PMA were unable to respond, in terms of increases in insulin release, to a 1 μ M-PMA stimulus during the subsequent perfusion. (2) As compared with the responses of control islets labelled in 2.75 mM-glucose alone, islets labelled in the additional presence of 1 μ M-PMA displayed a significant impairment in phosphoinositide (PI) hydrolysis, but an enhancement of both first- and second-phase insulin secretion, in response to subsequent 20 mM-glucose stimulation. (3) Decreasing extracellular Ca²⁺ level to 0.1 mM and including the Ca²⁺-channel antagonist nitrendipine (0.5 μ M) along with 1 μ M-PMA during the [³H]inositol-labelling period did not alter the response of the islets to the subsequent addition of 20 mM-glucose. Glucose-induced PI hydrolysis was still inhibited and 20 mM-glucose-induced insulin release was still enhanced. (4) A markedly amplified and sustained insulin-secretory response to 200 μ M-tolbutamide in the presence of 2.75 mM-glucose was also obtained from 1 μ M-PMA-pretreated islets. This contrasts sharply with the small and transient response to tolbutamide noted in control islets. (5) When present only during the perfusion phase of the experiments, nitrendipine (0.5 μ M) abolished the amplified insulin-secretory responses to both 20 mM-glucose and 200 μ M-tolbutamide noted in PMA-pretreated islets. (6) Prior labelling in 1 μ M-PMA dramatically amplified the insulinotropic effect of 25 mM-K⁺ or 5 μ M-A23187 stimulation. The amplified insulin-secretory response to K⁺, but not to A23187, was abolished by inclusion of nitrendipine during the perfusion. (7) Labelling in 1 μ M-PMA also decreased PI hydrolysis in response to cholecystokinin (CCK-8S) stimulation. (8) A 2 h pre-exposure to 1 μ M-PMA resulted in the persistent translocation of immunoreactive PKC to the membrane fraction. These results support the following conclusions. PMA pretreatment desensitizes the islet, in terms of agonist-induced increases in PI hydrolysis, to glucose, CCK-8S or tolbutamide. In spite of this inhibitory effect on PI hydrolysis, prior exposure to PMA dramatically amplified the insulin-secretory response to subsequent agonist stimulation. It is suggested that the chronic activation of PKC by PMA induces feedback inhibition of PI hydrolysis. However, the sensitivity of PKC activation to Ca²⁺, a result of persistent PKC membrane translocation, is dramatically increased. This accounts for the amplified insulin-secretory response despite diminished PI hydrolysis. These findings emphasize the importance of PKC activation in glucose-induced insulin secretion.

INTRODUCTION

The role of protein kinase C (PKC) activation in the sequence of events leading to glucose-induced insulin release remains controversial [1–4]. There is little doubt, however, that addition of a stimulatory glucose level increases phosphoinositide (PI) hydrolysis in islets [5–7] and thus generates the endogenous activator of PKC, diacylglycerol [8]. In addition, the phorbol ester phorbol 12-myristate 13-acetate (PMA; ‘TPA’), an established exogenous activator of PKC, induces a glucose-independent increase in insulin release and also sensitizes islets to subsequent glucose stimulation [9–11]. Both PMA and glucose also appear to cause the phosphorylation of similar protein substrates in islets [12–14], and both have been shown to enhance PKC translocation [15]. Furthermore, inhibitors of PKC have been shown to interfere with both glucose- and PMA-induced

insulin release [3,4]. On the other hand, under conditions thought to be accompanied by PKC depletion or inactivation (i.e. an 18–24 h pretreatment with PMA), it has been reported that glucose-induced insulin release is not inhibited [1,2]. On the basis of these findings, it has been concluded that PKC activation is not essential for glucose-dependent insulin release [1,2].

The present experiments were conducted to determine the influence of a 2 h exposure to the phorbol ester PMA on the subsequent activation of islets by PMA, glucose, tolbutamide, cholecystokinin (CCK-8S), high K⁺ and the Ca²⁺ ionophore A23187. Because PMA has been shown to impair the agonist-induced activation of PI-specific phospholipase C in other tissues [16,17], both PI hydrolysis and insulin release were examined. In addition, PMA-induced changes in PKC distribution were also assessed. Our findings emphasize the complex effects of PMA pretreatment on the subsequent responses of the β -cell.

Abbreviations used: PKC, protein kinase C; PI, phosphoinositide; PMA, phorbol 12-myristate 13-acetate (‘TPA’); CCK-8S, cholecystokinin.

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Fig. 1. Influence of prior 2 h exposure to PMA ($1 \mu\text{M}$) on subsequent responsiveness to PMA

Three groups of islets were studied. One group was labelled for 2 h in a [^3H]inositol-containing solution plus 2.75 mM-glucose ($G_{2.75}$) ($\circ$). A second group ($\bullet$) was exposed to $1 \mu\text{M}$ -PMA during this 2 h period. After washing, both groups were perfused for 30 min with 2.75 mM-glucose alone and for an additional 30 min with 2.75 mM-glucose plus $1 \mu\text{M}$ -PMA. A third group ($\bullet$, dashed line) of islets was labelled in the presence of $1 \mu\text{M}$ -PMA, washed and then perfused for 30 min with 2.75 mM-glucose, and then for an additional 30 min with 100 nM-staurosporine. This and subsequent Figures have been corrected for the dead space in the perfusion apparatus, 2.5 ml or 2.5 min with a flow rate of 1 ml/min. At least three experiments were performed under each experimental condition. *Significant ($P < 0.05$) difference, first versus second group.

MATERIALS AND METHODS

Male Sprague-Dawley rats purchased from Charles River were used in all studies. The animals were fed *ad lib.* and weighed 300–400 g. After Nembutal-induced (50 mg/kg) anaesthesia, islets were isolated by collagenase digestion [18]. Some islets were directly perfused. Other batches of islets (20–35) were loaded on to nylon filters and placed in small glass vials. They were incubated for 2 h in 200 μl of a *myo*-[2- ^3H]inositol-containing solution prepared by adding 10 μl of *myo*-[2- ^3H]inositol (initial sp. radioactivity 16.6–22.8 Ci/mmol) to 250 μl of incubation medium. The medium used for this incubation procedure was similar to that employed during the islet perfusion, and consisted of 115 mM-NaCl, 5 mM-KCl, 2.2 mM- CaCl_2 , 1 mM- MgCl_2 , 24 mM- NaHCO_3 and 0.17 g of BSA/dl. The solution was gassed with O_2/CO_2 (19:1). Glucose (2.75 mM) was also present during the incubation. In some experiments, 0.1 μM - or 1 μM -PMA was also present. PMA was dissolved in dimethyl sulphoxide. The amount of dimethyl sulphoxide (0.1%, v/v) used during the

incubation had no effect on the subsequent responses of perfused islets (W. S. Zawalich & K. C. Zawalich, unpublished work). After termination of the incubation, the islets still attached to the nylon filters, were washed with 5 ml of non-radioactive medium, and some were perfused. The pH of the perfusion medium was maintained at 7.4, the temperature at 37°C , and the flow rate at 1 ml/min. Islets were usually perfused for 30 min to establish stable insulin-secretory rates and then exposed to various agonists indicated in the Figure legends. Perfusate samples were collected at time intervals indicated in the Figures, and 200 μl samples were analysed for [^3H]inositol content as well as for insulin, with rat insulin (Lilly, no. 615-D63-12-3) as standard. Other islets were statically incubated to measure inositol phosphate accumulation. LiCl (10 mM) was used only in these static incubation experiments. Inositol phosphates were extracted after the perfusion or static incubation with 10% (w/v) HClO_4 and separated on anion-exchange columns (AG1-X8; 200–400 mesh; formate form; Bio-Rad, Richmond, CA, U.S.A.) by methods previously described [6,19]. These columns were prepared by adding (to give a length of 3 cm) anion-exchange resin to Pasteur pipettes. Further additions to the column included 10 ml of water and 5 ml of 5 mM- $\text{Na}_2\text{B}_4\text{O}_7/60$ mM-sodium formate. Elution of the inositol phosphates was accomplished by the sequential addition of 10 ml of 0.1 M-formic acid/0.2 M-ammonium formate (inositol 1-phosphate, InsP_1), 10 ml of 0.1 M-formic acid/0.4 M-ammonium formate (inositol 1,4-bisphosphate, InsP_2) and 10 ml of 0.1 M-formic acid/1 M-ammonium formate (inositol 1,4,5-trisphosphate plus inositol 1,3,4-trisphosphate, InsP_3). Samples (0.4 ml) of the eluate were then analysed for radioactive contents. Cellular content of radioisotope after inositol phosphate extraction was also determined. When total [^3H]inositol incorporation was determined, this was calculated as the sum of [^3H]inositol effluxing from the cell during min 28–60 of the perfusion, plus total [^3H]inositol phosphate accumulation, plus the amount of label remaining in the islets after [^3H]inositol phosphate extraction.

In some experiments, islets were incubated for 2 h in $1 \mu\text{M}$ -PMA. After washing, they were perfused for 30 min with 2.75 mM-glucose. After their retrieval they were sonicated, and membrane and cytosolic fractions were examined by quantitative immunoblotting for the presence of PKC. The detailed methodology for this procedure has been described in detail elsewhere [15]. Briefly, we have raised antibodies to the α , β and γ isoforms of PKC. Islets were sonicated in ice-cold buffer (20 mM-Tris, pH 7.4, 0.5 mM-EGTA, 50 μg of leupeptin/ml, 1 mM-phenylmethanesulphonyl fluoride, 0.1% β -mercaptoethanol, 25 μg of aprotinin/ml and 10 μM -pepstatin A), and centrifuged in a Beckman Airfuge [172 kPa (25 lb/in 2) for 10 min]. The supernatant was collected as the cytosolic fraction and the pellet collected as the membrane fraction. The fractions were then processed as described by immunoblotting [15]. Since the α form of PKC predominates in islets [15,20], only the distribution of α -PKC was monitored. The radiochemical used to measure insulin release (^{125}I -insulin) was purchased from New England Nuclear, Boston, MA, U.S.A., and the *myo*-[2- ^3H]inositol from Amersham, Arlington Heights, IL, U.S.A. PMA, BSA, CCK-8S (the C-terminal 8 amino acid residue sulphated on the tyrosine), as well as the salts used to make the perfusion medium, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Tolbutamide (sodium salt) was generously given by the Upjohn Co., Kalamazoo, MI, U.S.A. The Ca^{2+} ionophore A23187 was obtained from Calbiochem, San Diego, CA, U.S.A. Nitrendipine was generously given by Dr. A. Scriabine, Miles Institute for Preclinical Pharmacology, West Haven, CT, U.S.A. Staurosporine (dissolved in dimethyl sulphoxide) was obtained from Kyowa Hakko, U.S.A., Inc., New York, NY, U.S.A. Where

Table 1. Influence of labelling in PMA on inositol phosphate accumulation in batch-incubated islets

Groups of 20–30 islets were labelled for 2 h in a *myo*-[2-³H]inositol solution supplemented with 2.75 mM-glucose. PMA (1 μ M) and nitrendipine (Nitr; 0.5 μ M) were included in some experiments. After washing, the islets were stimulated with 2.75 mM-glucose ($G_{2.75}$) or 20 mM-glucose (G_{20}). Inositol phosphate accumulation was assessed. LiCl (10 mM) was included during this 40 min stimulation period. At least four experiments were conducted under each condition; results are means $\pm$ S.E.M. Statistical analysis: line 1 versus line 2, line 3 versus line 4, line 3 versus line 5, line 3 versus lines 4 and 5, all significant for all inositol phosphates.

Labelling conditions (min)	Incubation conditions (min)	Accumulation (c.p.m./40 islets)		
		InsP ₁	InsP ₂	InsP ₃
1. $G_{2.75}$ (120)	$G_{2.75}$ + LiCl (40)	2574 $\pm$ 145	233 $\pm$ 36	129 $\pm$ 26
2. $G_{2.75}$ (120)	$G_{2.75}$ + LiCl $\rightarrow$ G_{20} + LiCl (10) (30)	13 696 $\pm$ 1403	1299 $\pm$ 126	673 $\pm$ 87
3. $G_{2.75}$ + 1 μ M-PMA (120)	$G_{2.75}$ + LiCl (40)	2333 $\pm$ 440	245 $\pm$ 55	138 $\pm$ 37
4. $G_{2.75}$ + 1 μ M-PMA (120)	$G_{2.75}$ + LiCl $\rightarrow$ G_{20} + LiCl (10) (30)	9453 $\pm$ 595	715 $\pm$ 71	365 $\pm$ 37
5. $G_{2.75}$ + PMA + Nitr (120)	$G_{2.75}$ + LiCl $\rightarrow$ G_{20} + LiCl (10) (30)	7845 $\pm$ 719	536 $\pm$ 38	312 $\pm$ 92

Fig. 2. Influence of a 2 h exposure to PMA on [³H]inositol efflux rates and insulin secretion in response to a subsequent 20 mM-glucose stimulus

After labelling for 2 h in 2.75 mM-glucose ($G_{2.75}$) alone (○) or in PMA (Δ , 0.1 μ M; ●, 1 μ M), groups of islets were perfused for 30 min with 2.75 mM-glucose. For the next 30 min they were stimulated with 20 mM-glucose (G_{20}). Perfusate samples were collected and analysed for [³H]inositol, presented as fractional efflux rates (a), and insulin content (b). Means $\pm$ selected S.E.M. values of at least four experiments are given.

appropriate, statistical significance was determined by Student's *t* test for unpaired data or ANOVA in conjunction with the Neuman-Keuls test: $P < 0.05$ taken as significant. Unless otherwise stated, values presented in the Figures represent means $\pm$ S.E.M. of at least 3 observations.

RESULTS

Effect of PMA on insulin secretion

In freshly isolated islets, the addition of 1 μ M-PMA caused a delayed but significant increase in insulin secretion. After 60 min

of stimulation, insulin release in the presence of PMA averaged 308 ± 53 pg/min per islet (mean $\pm$ S.E.M., $n = 4$). Release from control islets, perfused in the presence of 2.75 mM-glucose alone, averaged only 22 ± 4 pg/min per islet at this time (results not shown). Decreasing Ca^{2+} to 0.1 mM and the further inclusion of 0.5 μ M-nitrendipine decreased, but did not completely abolish, the insulinotropic effect of 1 μ M-PMA. Under these conditions, PMA induced an approx. 2-fold increase in the insulin-secretory rate.

Islets labelled with [³H]inositol in the presence of 1 μ M-PMA for 2 h displayed a significantly impaired secretory response to

Table 2. Influence of labelling in PMA on inositol phosphate accumulation in isolated perfused islets

Groups of 28–35 islets were labelled for 120 min with [^3H]inositol in the presence of 2.75 mM-glucose ($G_{2.75}$) alone or in the additional presence of PMA (0.1 or 1 μM). After washing to remove unincorporated label, the islets were perfused for 30 min in 2.75 mM-glucose. In some islets the perfusion was continued for an additional 30 min with this glucose level. In others, the perfusion level of glucose was increased to 20 mM (G_{20}). Other groups were stimulated with tolbutamide (Tol; 200 μM) or CCK-8S (200 nM). At the termination of the perfusion, levels of inositol phosphates (IPs) were measured. At least four experiments were conducted under each condition; results are means $\pm$ S.E.M. Statistical analysis: line 1 versus line 2, significant for all IPs; line 1 versus line 3, significant for InsP_2 ; line 2 versus line 3, significant for all IPs; line 2 versus line 4, no significant differences; line 1 versus line 5, no significant differences; line 2 versus line 6, significant for all IPs; line 5 versus line 6, significant for all IPs; line 7 versus line 8, significant for InsP_1 ; line 9 versus line 10, significant for all IPs.

Labelling conditions (min)	Perfusion conditions (min)	Accumulation (c.p.m./40 islets)		
		InsP_1	InsP_2	InsP_3
1. $G_{2.75}$ (120)	$G_{2.75}$ (60)	533 $\pm$ 75	118 $\pm$ 19	72 $\pm$ 16
2. $G_{2.75}$ (120)	$G_{2.75} \rightarrow G_{20}$ (20) (30)	1455 $\pm$ 127	639 $\pm$ 72	216 $\pm$ 26
3. $G_{2.75} + 0.1 \mu\text{M-PMA}$ (120)	$G_{2.75}$ (60)	631 $\pm$ 48	212 $\pm$ 30	64 $\pm$ 8
4. $G_{2.75} + 0.1 \mu\text{M-PMA}$ (120)	$G_{2.75} \rightarrow G_{20}$ (60) (30)	1301 $\pm$ 115	545 $\pm$ 120	190 $\pm$ 42
5. $G_{2.75} + 1 \mu\text{M-PMA}$ (120)	$G_{2.75}$ (60)	698 $\pm$ 73	172 $\pm$ 41	76 $\pm$ 19
6. $G_{2.75} + 1 \mu\text{M-PMA}$ (120)	$G_{2.75} \rightarrow G_{20}$ (30) (30)	949 $\pm$ 105	285 $\pm$ 28	139 $\pm$ 22
7. $G_{2.75}$ (120)	$G_{2.75} \rightarrow G_{2.75} + \text{Tol}$ (30) (30)	717 $\pm$ 43	136 $\pm$ 21	92 $\pm$ 18
8. $G_{2.75} + 1 \mu\text{M-PMA}$ (120)	$G_{2.75} \rightarrow G_{2.75} + \text{Tol}$ (30) (30)	584 $\pm$ 37	126 $\pm$ 15	82 $\pm$ 14
9. $G_{2.75}$ (120)	$G_{2.75} \rightarrow G_{2.75} + \text{CCK}$ (30) (30)	2628 $\pm$ 198	955 $\pm$ 107	238 $\pm$ 36
10. $G_{2.75} + 1 \mu\text{M-PMA}$ (120)	$G_{2.75} \rightarrow G_{2.75} + \text{CCK}$ (30) (30)	1854 $\pm$ 213	531 $\pm$ 73	102 $\pm$ 14

subsequent stimulation with 1 $\mu\text{M-PMA}$. In these islets (Fig. 1), the basal insulin-release rate, in the presence of 2.75 mM-glucose, was elevated 3–4-fold. Exposure to 1 $\mu\text{M-PMA}$ during the subsequent perfusion did not induce a significant insulin-secretory response from these islets. In contrast, islets labelled with [^3H]inositol in 2.75 mM-glucose alone for 2 h responded to 1 $\mu\text{M-PMA}$ with a significant insulin-secretory response (Fig. 1). Of particular note was the observation that the addition of 100 nM of the PKC inhibitor staurosporine to PMA-pretreated islets decreased the basal insulin-secretory rates of these islets to control values (Fig. 1).

Effect of PMA on [^3H]inositol incorporation and [^3H]inositol phosphate production

The impact of PMA on the incorporation of [^3H]inositol was examined by incubating islets in [^3H]inositol, with or without 0.1 $\mu\text{M-}$ or 1 $\mu\text{M-PMA}$, for a period of 2 h. Total [^3H]inositol uptake in the presence of 0.1 $\mu\text{M-}$ (28 808 $\pm$ 2771 c.p.m., $n = 8$) or 1 $\mu\text{M-}$ (30 617 $\pm$ 1902, $n = 12$) PMA was comparable with that observed in control islets labelled in 2.75 mM-glucose alone (25 123 $\pm$ 1769, $n = 12$). After labelling in the presence or absence of 1 $\mu\text{M-PMA}$, the accumulation of inositol phosphates under various conditions was determined in static batch incubations in the presence of Li^+ . In the presence of 2.75 mM-glucose, inositol phosphate accumulations were modest (Table 1, lines 1 and 3). In the presence of 20 mM-glucose, however, large and significant increases in the amounts of these compounds were noted (line 2). When stimulated with 20 mM-glucose, islets prelabelled in the presence of 1 $\mu\text{M-PMA}$ displayed an impairment in the

accumulation of these compounds (line 4). In additional experiments, the Ca^{2+} level used during the 2 h incubation period was decreased to 0.1 mM (the normal Ca^{2+} level used is 2.2 mM), and the Ca^{2+} -channel blocker nitrendipine (0.5 μM) was also included. These changes did not prevent the inhibitory effect of PMA on the subsequent inositol phosphate responses to 20 mM-glucose (line 5).

Effect of PMA pretreatment on [^3H]inositol efflux, [^3H]inositol phosphate accumulation and insulin release in perfused islets stimulated with 20 mM-glucose

Islets were labelled with [^3H]inositol for 2 h in 2.75 mM-glucose alone or with the further addition of 0.1 $\mu\text{M-}$ or 1 $\mu\text{M-PMA}$. After washing with PMA-free medium they were perfused with 2.75 mM-glucose to measure basal rates of [^3H]inositol efflux and insulin secretion. After this control period the responses to 20 mM-glucose were determined. As shown in Fig. 2(a), control islets labelled in 2.75 mM-glucose alone responded to 20 mM-glucose with significant increases in [^3H]inositol efflux. They also exhibited a biphasic insulin-secretory response (Fig. 2b). When labelled in the presence of 0.1 $\mu\text{M-PMA}$, the subsequent addition of 20 mM-glucose led to nearly the same increase in [^3H]inositol efflux as seen in control islets (Fig. 2). However, the islets preincubated in 0.1 $\mu\text{M-PMA}$ displayed enhanced first- and second-phase insulin release (Fig. 2). When the PMA concentration was 1 μM during the 2 h preincubation period, the subsequent addition of 20 mM-glucose led to only a small and delayed increase in [^3H]inositol efflux but, paradoxically, an even more dramatic amplification of both first- and second-

Fig. 3. Influence of labelling in 1 μ M-PMA on subsequent insulin-secretory responses to 200 μ M-tolbutamide

After labelling in 2.75 mM-glucose with (●) or without (○) 1 μ M-PMA, groups of islets were perfused for 60 min with 2.75 mM-glucose ($G_{2.75}$). For the final 30 min 200 μ M-tolbutamide (TOL) was also included in the perfusate. In one set of PMA-pretreated islets, 0.5 μ M-nitrendipine (Nitr) was included 10 min before and during the perfusion period with tolbutamide (■, dashed line). At least three experiments were performed under each condition.

phase insulin release. Basal insulin-secretory rates observed in the presence of 2.75 mM-glucose were also elevated after labelling in 1 μ M-PMA.

When measured at the termination of the perfusion, it was also found that labelling in 1 μ M-PMA, but not 0.1 μ M, significantly decreased [3 H]inositol phosphate accumulation in response to subsequent stimulation with 20 mM-glucose (Table 2, lines 4 and 6).

Effect of PMA pretreatment on insulin secretion in response to tolbutamide

In control islets labelled in the presence of 2.75 mM-glucose alone, the sulphonylurea tolbutamide (200 μ M) evoked a small increase in insulin release from islets perfused in the presence of 2.75 mM-glucose (Fig. 3). However, in PMA-pretreated islets, addition of the sulphonylurea evoked a dramatic and sustained biphasic insulin-secretory response (Fig. 3). This effect of tolbutamide was completely inhibited by addition of 0.5 μ M-nitrendipine (Fig. 3). Prior PMA pretreatment also decreased the small effect of 200 μ M-tolbutamide (in the presence of 2.75 mM-glucose) on inositol phosphate accumulation (Table 2, lines 7 and 8).

Effect of Ca^{2+} deprivation during the 2 h labelling period on PMA-induced sensitization of insulin secretion

Recently, Regazzi and co-workers [21] reported that PMA induces a Ca^{2+} -independent activation of PKC in RINm5F cells.

Fig. 4. Influence of nitrendipine (0.5 μ M) on glucose-induced insulin secretion from PMA-pretreated islets

Two groups of islets were exposed for 2 h to [3 H]inositol plus 2.75 mM-glucose ($G_{2.75}$) plus 1 μ M-PMA. They were then perfused for 60 min. For the final 30 min they were stimulated with 20 mM-glucose (G_{20}) with or without nitrendipine (Nitr). The nitrendipine was added to the medium 10 min before 20 mM-glucose. At least three experiments were performed under each condition.

Moreover, our results (see above and Table 1) suggest that PMA-induced insulin release and the inhibitory effect of PMA on glucose-induced PI hydrolysis are to some extent Ca^{2+} -independent. We examined whether the potentiation of insulin release induced by prior exposure to PMA is similarly Ca^{2+} -independent. In these studies, the Ca^{2+} level during the 2 h labelling period was decreased to 0.1 mM and the Ca^{2+} -channel blocker nitrendipine (0.5 μ M) was also included. After the 2 h incubation, these islets were perfused with a medium containing 2.75 mM-glucose, a normal Ca^{2+} concentration (2.2 mM) and without the Ca^{2+} -channel blocker. Pretreatment of islets with PMA under these conditions had no significant effect on the ability of PMA to sensitize islets to a subsequent challenge with 20 mM-glucose (results not shown).

Effect of decreasing Ca^{2+} influx during the perfusion on insulin release in response to 20 mM-glucose and tolbutamide from PMA-sensitized islets

A reasonable explanation for the markedly amplified insulin responses to 20 mM-glucose noted after PMA pre-exposure may be that the Ca^{2+} -sensitivity of PKC is increased. For example, translocation of PKC to the membrane is thought to improve the Ca^{2+} -dependent catalytic activity of the enzyme. The next set of experiments was conducted to determine whether decreasing Ca^{2+} influx into the β -cell abrogates the amplified insulin responses to 20 mM-glucose in PMA-pretreated islets. These results, given in Figs. 3 and 4, show that the inclusion of

Fig. 5. Influence of 1 μ M-PMA on 25 mM- K^+ -induced insulin secretion

Three groups of islets were studied. One group (○) was labelled for 2 h in a [3 H]inositol-containing solution supplemented with 2.75 mM-glucose. The other groups (●) were labelled in the additional presence of 1 μ M-PMA. After washing, the islets were perfused for 30 min with 2.75 mM-glucose ($G_{2.75}$) and for an additional 30 min with 25 mM- K^+ . In one of the PMA-pretreated groups (●, dashed lines), 0.5 μ M-nitrendipine (Nitr) was included during the perfusion. The Ca^{2+} -channel blocker was present 10 min before the 25 mM- K^+ stimulation. At least three experiments were performed under each condition.

Fig. 6. Influence of labelling in 1 μ M-PMA on A23187-induced insulin secretion

Three groups of islets were studied. One group (○) was labelled for 2 h in [3 H]inositol plus 2.75 mM-glucose. The other two groups were similarly treated except for the additional presence of 1 μ M-PMA. After 2 h, the islets were washed and perfused for 30 min with 2.75 mM-glucose ($G_{2.75}$) and for an additional 30 min with the same glucose level plus 5 μ M-A23187. In one of the PMA-pretreated groups, nitrendipine (Nitr; 0.5 μ M) was included during the final 40 min of the perfusion. At least three experiments were conducted under each experimental condition.

nitrendipine (0.5 μ M) during the perfusion significantly decreases the responsiveness of these sensitized PMA-pretreated islets to subsequent stimulation with glucose (Fig. 4) or tolbutamide (Fig. 3).

Effects of 1 μ M-PMA pretreatment on K^+ (25 mM)- and A23187-stimulated insulin secretion

Further studies were conducted after islets were incubated for 2 h in 2.75 mM-glucose with or without 1 μ M-PMA. In control islets incubated in 2.75 mM-glucose alone, 25 mM- K^+ (Fig. 5) or 5 μ M of the Ca^{2+} ionophore A23187 (Fig. 6) stimulated insulin secretion during a subsequent perfusion. Under the same perfusion conditions, a prior 2 h exposure to 1 μ M-PMA dramatically amplified these insulin-secretory responses. Furthermore, the presence of the Ca^{2+} -channel blocker nitrendipine (0.5 μ M) decreased the amplified insulin-secretory response to K^+ but not to A23187 (Figs. 5 and 6).

Effect of PMA pretreatment on cholecystokinin (CCK-8S)-induced PI hydrolysis

To test the specificity of PMA-induced decreases in PI hydro-

lysis, experiments were conducted with CCK-8S as agonist. Islets were again [3 H]inositol-labelled for 2 h in the presence of 2.75 mM-glucose with or without 1 μ M-PMA. After washing and a 30 min perfusion with 2.75 mM-glucose alone, these islets were stimulated with 200 nM-CCK-8S. Labelling in the presence of PMA significantly decreased the increase in [3 H]inositol efflux usually noted in response to CCK-8S (Fig. 7a). Furthermore, labelling in PMA also decreased inositol phosphate formation in these islets (Table 2, lines 9 and 10). A slight increase in CCK-8S-induced insulin secretion was also observed (Fig. 7b).

Effect of PMA on PKC distribution

In the final series of experiments, groups of islets were incubated for 2 h in the presence or absence of 1 μ M-PMA. They were then perfused for 30 min with 2.75 mM-glucose. After retrieval, the islets were sonicated, and distribution of immunoreactive PKC was assessed by quantitative immunoblotting. In control islets, approx. 70% of the PKC immunoreactivity was associated with the cytoplasmic compartment (Fig. 8). However, after pretreatment with 1 μ M-PMA (and an intervening 30 min perfusion in the absence of PMA), approx. 70% of the PKC immunoreactivity was then membrane-associated (Fig. 8).

Fig. 7. Influence of labelling in PMA ($1 \mu\text{M}$) on CCK-8S-induced increases in [^3H]inositol efflux rates and insulin secretion

Groups of islets were labelled for 2 h in 2.75 mM-glucose with or without $1 \mu\text{M}$ -PMA. They were then washed and perfused for 60 min with 2.75 mM-glucose ($G_{2.75}$). For the final 30 min of this period, 200 nM-CCK-8S was included in the medium. Fractional rates of [^3H]inositol efflux (a) and insulin output (b) were measured. At least three experiments were performed under each condition. *Significant difference ($P < 0.05$).

Fig. 8. Influence of PMA ($1 \mu\text{M}$) on PKC distribution

Two groups of islets were incubated for 2 h with or without $1 \mu\text{M}$ -PMA. Both groups were then perfused for 30 min with 2.75 mM-glucose ($G_{2.75}$) alone. The islets were then sonicated, separated into membrane and cytosol fractions by centrifugation in a Beckman Airfuge [172 kPa (25 lb/in 2) for 10 min] and processed by immunoblotting with an α -PKC antibody. The 80 kDa α -PKC band was quantified on a Visage 2000 system. Results are expressed as $100 \times (\text{optical density of an } \alpha\text{-PKC band in the fraction}) / (\text{total optical density})$, where the total optical density is the sum of the optical density of the α -PKC band in the cytosol fraction plus the optical density (O.D.) of the α -PKC band in the membrane fraction. Data are shown as means $\pm$ S.E.M. ($n = 4$ for each condition; $P < 0.03$ for islets incubated with $G_{2.75}$ alone versus $G_{2.75}$ plus $1 \mu\text{M}$ -PMA). A typical immunoblot showing the relative membrane (M)/cytosol (C) distribution of α -PKC is shown below the histogram for each experimental condition. Values to the left of the immunoblots refer to molecular masses (kDa) of prestained standards.

DISCUSSION

Labelling in the presence of PMA had no adverse effect on the total amount of [^3H]inositol incorporated by the islets. Although most (80–90%) of this label is present in the lipid fraction, we did not examine whether PMA influenced the interconversions of the various inositol-containing phospholipids. However, it should be noted that in the presence of 2.75 mM-glucose the basal levels of the various inositol phosphates measured were virtually identical in control and PMA-pretreated islets (see Table 1, lines 1 and 3, and Table 2, lines 1 and 5).

Preincubation in $1 \mu\text{M}$ -PMA for 2 h results in higher basal rates of insulin release during the subsequent perfusion. Insulin secretion in response to a subsequent stimulation with glucose, tolbutamide, K^+ or ionophore is dramatically enhanced. These enhanced responses may be a consequence of the fact that, once associated with the membrane, PKC in cells pretreated with PMA does not rapidly dissociate. This conclusion is supported by our α PKC measurements. After PMA pretreatment, greater than 70% (compared with less than 30% in controls) of the PKC immunoreactivity is membrane-associated.

Of particular note is the fact that, after labelling in $1 \mu\text{M}$ -PMA for 2 h, islets lose their ability to respond, at least in terms of insulin release, to a subsequent PMA stimulus applied during the perfusion (Fig. 1). Nonetheless, their ability to respond, again in terms of insulin release, to a wide variety of structurally diverse stimulants is markedly amplified (Figs. 2, 3, 5 and 6). After incubation in $1 \mu\text{M}$ -PMA, peak first-phase secretion to 20 mM-glucose is approx. 15-fold greater than that observed from control islets. Second-phase release is also amplified, approx. 3-fold.

It is noteworthy that in two previous studies where islet secretory responses to glucose were measured after 18–24 h of PMA pretreatment, an amplified insulin-secretory response to glucose was also observed [1,2]. However, in these studies, as in the present one, the insulin-secretory response to PMA itself was lost. Because of this dissociation, it was concluded that PKC is not involved in glucose-induced insulin secretion [1,2]. We disagree with this conclusion for several reasons. First, as shown in this and other studies [10,11], prior PMA exposure has dramatic and sustained effects on insulin secretion, effects presumably mediated by PKC itself or some product generated by its activation. Second, in the study by Hii *et al.* [2], their control islets increased their release of insulin less than 2-fold above basal rates observed with 5.6 mM-glucose. In contrast, the normal response is at least a 10–20-fold increase (Fig. 2; see also ref. [22]). It is difficult to come to any conclusion concerning involvement of PKC in the physiological regulation of insulin secretion in islets already so profoundly impaired. Third, the inability of PMA to evoke secretion from PMA-pretreated islets occurs rapidly (Fig. 1) and at a time when β -cell responsiveness to other agonists is not impaired, but actually enhanced. It is noteworthy that, under this circumstance, the basal rate of insulin secretion is enhanced (Fig. 1) and that this effect on basal release is abolished by staurosporine (Fig. 1). In addition, the inability of PMA to evoke secretion from PMA-pretreated islets may be due to the failure of PMA to influence other second-messenger systems involved in stimulated secretion. For example, PMA is known to influence β -cell Ca^{2+} handling and cyclic AMP accumulation [9]. If one or both of these actions of PMA were impaired by prior exposure to the phorbol ester, then the insulinotropic effect of PMA itself might be abrogated.

Finally, the involvement of PKC in glucose-induced insulin secretion by 'PKC-depleted' islets has been examined in detail by Thams and co-workers [23]. In the previous 'depletion' studies, release was measured under batch-incubation conditions [1,2], a

methodology that yields no information concerning the dynamic biphasic insulin-secretory response. Thams and co-workers [23], however, perfused their islets. They concluded that PKC activation was involved in the second phase of glucose-induced insulin secretion. They also reported that 5–15% of PKC activity remained even after attempts to deplete the islet of PKC by culturing in PMA, a finding similar to the PKC-depletion studies of Arkhammar *et al.* [14]. The present results indicate that short-term (2 h) PMA pretreatment results in a sustained increase in the β -cell sensitivity to a variety of agonists, a situation that occurs and persists despite the inability of PMA itself to provoke secretion.

What biochemical alteration is induced by PMA to account for such a dramatic increase in 20 mM-glucose-induced release, an increase that manifests itself despite an actual decrease in 20 mM-glucose-induced PI hydrolysis? A reasonable suggestion, and one confirmed by α PKC measurements, is that PMA pretreatment promotes the translocation of PKC to the membrane, an event that not only persists for a long period of time but also increases the Ca^{2+} -sensitivity of the enzyme [21,24,25]. This concept is also consistent with previous studies demonstrating that PMA promotes PKC translocation [15,24] and sensitizes the islet to subsequent stimulation with glucose [10,11], and also with our results obtained with tolbutamide in the presence or absence of nitrendipine (Fig. 3). Although tolbutamide evokes only a small response from control islets, pretreatment with PMA amplifies and sustains this response. Considering the fact that the major effect of the sulphonylurea is to increase Ca^{2+} influx into the cell [26], this event might be expected to increase dramatically the Ca^{2+} -dependent catalytic activity of membrane-translocated PKC. The fact that nitrendipine abolishes this amplified response not only to the sulphonylurea but also to glucose underscores further the important role played by Ca^{2+} influx in this amplification process.

Further support for the essential involvement of Ca^{2+} influx in these amplified insulin-secretory responses comes from our studies with high- K^+ or Ca^{2+} -ionophore stimulation after PMA pretreatment. In these studies, each agonist evoked a dramatic increase in insulin release from PMA-pretreated islets, but only a modest response from control islets. Of paramount importance, however, was the further finding that blocking Ca^{2+} influx into the β -cell with nitrendipine virtually abolished the amplified insulin-secretory response to K^+ but not to A23187. Since not only K^+ , but also glucose and tolbutamide, increase Ca^{2+} influx via L-type Ca^{2+} channels, nitrendipine would be expected to decrease Ca^{2+} influx in response to these agonists. However, A23187 by-passes these channels, and increases intracellular Ca^{2+} by creating its own 'channels'. Clearly, experiments of this type point to a markedly increased sensitivity to Ca^{2+} of the transduction mechanism involved in regulating insulin secretion. It is reasonable to conclude that anchoring of the enzyme PKC to the plasma membrane of these PMA-pretreated cells accounts for their enhanced responsiveness to Ca^{2+} influx. Whether our pretreatment protocol with PMA alters the mem-

brane potential, and thus indirectly influences Ca^{2+} influx, was not determined, but an effect on ion fluxes might also contribute to this amplification effect [27].

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