Potentiation of stimulus-induced insulin secretion in protein kinase C-deficient RINm5F cells

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The role of protein kinase C (PKC) in stimulus recognition and insulin secretion was investigated after long-term (24 h) treatment of RINm5F cells with phorbol 12-myristate 13-acetate (PMA). Three methods revealed that PKC was no longer detectable, and PMA-induced insulin secretion was abolished. Such PKC-deficient cells displayed enhanced insulin secretion (2–6-fold) in response to vasopressin and carbachol (activating phospholipase C) as well as to D-glyceraldehyde and alanine (promoting membrane depolarization and voltage-gated Ca^{2+} influx). Insulin release stimulated by 1-oleoyl-2-acetylglycerol (OAG) was also greater in PKC-deficient cells. OAG caused membrane depolarization and raised the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), both of which were unaffected by PKC down-regulation. Except for that caused by vasopressin, the secretagogue-induced $[Ca^{2+}]_i$ elevations were similar in control and PKC-depleted cells. The $[Ca^{2+}]_i$ rise evoked by vasopressin was enhanced during the early phase (observed both in cell suspensions and at the single cell level) and the stimulation of diacylglycerol production was also augmented. These findings suggest more efficient activation of phospholipase C by vasopressin after PKC depletion. Electrically permeabilized cells were used to test whether the release process is facilitated after long-term PMA treatment. PKC deficiency was associated with only slightly increased responsiveness to half-maximally (2 μM) but not to maximally stimulatory Ca^{2+} concentrations. At 2 μM-Ca^{2+} vasopressin caused secretion, which was also augmented by PMA pretreatment. The difference between intact and permeabilized cells could indicate the loss in the latter of soluble factors which mediate the enhanced secretory responses. However, changes in cyclic AMP production could not explain the difference. These results demonstrate that PKC not only exerts inhibitory influences on the coupling of receptors to phospholipase C but also interferes with more distal steps implicated in insulin secretion.

INTRODUCTION

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent enzyme which is activated by diacylglycerol (DAG) generated via breakdown of phosphoinositides and other phospholipids (Nishizuka, 1984, 1988; Berridge, 1987). PKC can also be directly activated by phorbol esters (Nishizuka, 1984, 1988) such as phorbol 12-myristate 13-acetate (PMA). There are at least seven isoforms of this enzyme which differ in terms of substrate specificity and their Ca^{2+} dependence (Nishizuka, 1988; Kikkawa *et al.*, 1989). PKC is down-regulated by long-term treatment with phorbol esters, which is mostly due to the degradation of membrane-bound activated PKC by the proteolytic enzymes such as calpain (Kikkawa *et al.*, 1989; Kishimoto *et al.*, 1989). The extent of down-regulation of PKC is dependent on the cell type and the isoform of the enzyme, the time of treatment and the phorbol ester concentration (Huang *et al.*, 1989; Adams & Gullick, 1989).

The role of PKC in cell activation in general and insulin secretion in particular is still unclear. Phorbol esters and cell-permeant DAGs enhance insulin release from both pancreatic islets and insulin-secreting cell lines (Virji *et al.*, 1978; Malaisse *et al.*, 1985; Wollheim *et al.*, 1988; Hughes *et al.*, 1990). Ca^{2+}-mobilizing neurotransmitters and hormones stimulate the generation of DAG (Peter-Riesch *et al.*, 1988; Wolf *et al.*, 1989; Regazzi *et al.*, 1990) and activate PKC in insulin-secreting cells (Yamatani *et al.*, 1988; Persaud *et al.*, 1989*a*; Arkhammar *et al.*, 1989; Regazzi *et al.*, 1990). However, these agents elicit only modest insulin release when added alone (Garcia *et al.*, 1988; Thams *et al.*, 1990; Regazzi *et al.*, 1990). The nutrient stimuli, glucose and D-glyceraldehyde, in contrast with Ca^{2+}-mobilizing compounds, primarily depolarize β cells and promote Ca^{2+} influx (Wollheim & Pozzan, 1984; Wollheim & Biden, 1986; Petersen & Findlay, 1987). Although these secretagogues affect the turnover of DAG by synthesis *de novo* from glycolytic intermediates (Peter-Riesch *et al.*, 1988; Wollheim *et al.*, 1988; Wolf *et al.*, 1989, 1990), this process appears to contribute only to a minor extent to cellular DAG levels (Wolf *et al.*, 1990; Regazzi *et al.*, 1990). An additional small contribution of DAG originating from phosphoinositides is due to activation of phospholipase C following Ca^{2+} entry in nutrient-stimulated cells (Best, 1986; Biden *et al.*, 1987). Thus far, several studies have failed to demonstrate involvement of PKC in glucose-stimulated insulin secretion either by indirect means or by direct measurement of PKC activity (Hii *et al.*, 1987; Metz, 1988; Persaud *et al.*, 1989*a*; Easom *et al.*, 1989; Arkhammar *et al.*, 1989; Regazzi *et al.*, 1990).

Although the muscarinic agonist carbachol has been shown to increase PKC activity in islets (Persaud *et al.*, 1989*a*; Arkhammar *et al.*, 1989), the agonist is still capable of causing part of its stimulatory effect on insulin secretion in PKC-down-regulated islets (Persaud *et al.*, 1989*b*; Thams *et al.*, 1990). These results prompted the conclusion that carbachol acts via both PKC-dependent and PKC-independent pathways. However, PKC activity was unchanged in the membrane fraction and incompletely down-regulated in the cytosolic fraction (Persaud *et al.*, 1989*b*) or in homogenates (Thams *et al.*, 1990) of the phorbol ester-treated islets. In many cell systems PKC has both stimulatory and inhibitory effects (Nishizuka, 1988; Kikkawa *et al.*, 1989). Therefore the delineation of PKC action when the down-regulation procedure is used depends on equally efficient elimination of both types of influences.

Abbreviations used: AVP, arginine-vasopressin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; DAG, 1,2-diacylglycerol; OAG, 1-oleoyl-2-acetylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; KRB, Krebs–Ringer buffer.

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In the present work, we have used down-regulation of PKC by PMA treatment of RINm5F cells in order to probe for the role of the enzyme in signal transduction and insulin release in response to agents which act by various mechanisms.

EXPERIMENTAL

Cell culture

RINm5F cells were cultured in RPMI 1640 medium and transferred to spinner culture for 3 h at 37 °C prior to the experiments, as described in detail previously (Wollheim & Pozzan, 1984).

Down-regulation of PKC

Cells were treated with 100 nM-PMA for 24 h in culture, and the spinner medium also contained this concentration of PMA. Control cells were treated in parallel with vehicle (0.1 % dimethyl sulphoxide).

Measurement of PKC

For PKC measurements, cells were disrupted by sonication in the homogenization buffer (20 mM-Tris/HCl, pH 7.4, 2 mM-EGTA, 2 mM-EDTA, 6 mM-mercaptoethanol, 0.25 M sucrose, 10 μg of leupeptin/ml and 2 μg of aprotinin/ml). The homogenate was centrifuged for 1 h at 100000 ***g***, resulting in a supernatant (cytosol) and a pellet (membranes). The latter was resuspended by sonication in 1 ml of homogenization buffer supplemented with 1 % Triton X-100 and centrifuged again for 1 h at 100000 ***g*** yielding a microsomal extract. Both cytosolic and membrane fractions were stored at −20 °C until use. After purification of PKC by polyacrylamide-gel electrophoresis, PKC activity was assayed by measuring ^{32}P incorporation from [γ-^{32}P]ATP into protamine sulphate as previously described (Fabbro *et al.*, 1986; Bruzzone *et al.*, 1988).

For detection of phorbol ester binding, the cytosolic and membrane fractions were incubated with 0.1 μCi of [^{3}H]phorbol dibutyrate (20 nM) in the presence of phosphatidylserine (100 μg) and Ca^{2+} (1 mM) (Costa *et al.*, 1985). Specific binding was calculated by subtracting non-specific binding (obtained with 200-fold excess unlabelled ligand) from total binding.

For the immunoblot analysis of PKC, the subcellular fractions were subjected to polyacrylamide-gel electrophoresis and the proteins were transferred electrophoretically to nitrocellulose membranes. The nitrocellulose sheets were blocked in TN buffer (50 mM-Tris/HCl, pH 7.4, 200 mM-NaCl, and 0.05 % Tween-20) containing 4 % BSA and incubated for 90 min with purified anti-PKC antibodies (1:50) (Erne *et al.*, 1987). Thereafter 0.2 μCi of ^{125}I-protein A/ml was added for 90 min. The nitrocellulose was then extensively washed with TN buffer and autoradiographed at −70 °C.

Measurement of cytosolic free calcium ($[Ca^{2+}]_i$)

For measurement of $[Ca^{2+}]_i$ in cell suspensions, cells were incubated in the presence of 1 μM-fura-2/acetoxymethyl ester for 30 min at 37 °C and washed with modified Krebs–Ringer bicarbonate buffer (KRB) containing 25 mM-Hepes, 2.8 mM-glucose, 1 mM-$CaCl_2$ and 5 mM-$NaHCO_3$ pH 7.4. $[Ca^{2+}]_i$ was estimated as described in detail previously (Wollheim *et al.*, 1988).

For measurement of $[Ca^{2+}]_i$ in single cells, cells grown on glass cover-slips were loaded for 20 min with 1 μM-fura-2/acetoxymethyl ester at 37 °C and fluorescence was measured by dual-wavelength (excitation 340/380 nm) microfluorimetry as described for single β cells (Pralong *et al.*, 1990).

Determination of DAG accumulation

Cells were labelled with 15 μCi of [2-^{3}H]glycerol/ml (1 Ci/mmol) in RPMI 1640 medium for 48 h. After washing with KRB, suspensions of about (3–5) × 10^6 cells in 0.5 ml of KRB were preincubated for 10 min at 37 °C. Thereafter, another 0.5 ml of KRB containing 2-fold-concentrated stimuli was added. The incubation was terminated by adding 6 ml of ice-cold methanol/chloroform (2:1, v/v). After addition of 2 ml of chloroform and 1.5 ml of water, the samples were vortex-mixed and extracted overnight at −20 °C. The aqueous phase was aspirated and the lipid phase was washed three times with 2 ml of chloroform/water (1:1, v/v). After drying under N_2, the samples were reconstituted in 55 μl of chloroform/methanol/water (75:25:2, by vol.) containing 25 μg each of mono-, di- and tri-acylglycerol standards. The extracted lipids were spotted on t.l.c. plates of silica 60 gel. The plates were developed in a mixture of benzene/ether/ethanol/acetic acid (25:20:1:0.1, by vol.). Neutral lipid spots were stained by iodine vapour and the spots identified as DAG were scraped into scintillation vials. Scintillant (10 ml) was added and radioactivity was counted (Peter-Riesch *et al.*, 1988).

Measurement of cyclic AMP levels

Cells were incubated as described for DAG measurements. After addition of boiling acetate buffer (0.2 M), the cell suspensions were boiled for 20 min. The samples were then centrifuged, the supernatants were acetylated and the measurements of cyclic AMP were performed by using a radioimmunoassay kit (Amersham International).

Assessment of insulin secretion

For measurement of insulin release, approx. 5 × 10^6 cells were placed in each of eight chambers of a perifusion apparatus (Kikuchi *et al.*, 1974). The perifusion medium was KRB containing 0.07 % BSA, and experiments were performed at 37 °C. The flow rate was 1 ml/min. The cells were first perifused for 45 min for stabilization of secretion rates before addition of stimuli. The collected samples were kept at −20 °C until assayed. Cellular insulin content was measured after acid/ethanol extraction as described previously (Wollheim & Pozzan, 1984).

Because the insulin content of cells was decreased after 24 h of PMA treatment (394.7 ± 48.2 and 247.8 ± 29.5 ng/10^6 cells in control and PMA-treated cells respectively; $n = 12$), insulin release was expressed as percentage of initial cellular hormone content.

For measurement of insulin release from electrically permeabilized cells, the cells were washed with modified Ca^{2+}-free KRB and resuspended in ice-cold mannitol buffer as described previously (Vallar *et al.*, 1987). The cells were rendered permeable by 10 exposures (30 μs each) to an electric field of 3 kV/cm at 4 °C. After centrifugation, the cells were resuspended in ice-cold potassium glutamate buffer (Vallar *et al.*, 1987). Portions of permeabilized cells (approx. 1 × 10^6 cells) were added to potassium glutamate buffer containing 10.2 mM-EGTA, different concentrations of Ca^{2+} and the test substances. After a 15 min preincubation at 4 °C, the tubes were incubated for 5 min at 37 °C. The incubation was stopped on ice. The samples were centrifuged and the supernatants were stored at −20 °C until assay.

Immunoreactive insulin was measured by radioimmunoassay using rat insulin as standard and guinea-pig anti-(pig insulin) serum kindly provided by Dr. F. Bousen, Novo-Nordisk, Gentofte, Denmark.

Measurement of membrane potential

Qualitative changes in membrane potential were assessed with

the fluorescent probe bisoxonol. About 2×10^6 cells were suspended in a cuvette and bisoxonol was added to yield a final concentration of 100 nM. When the fluorescence was at equilibrium the stimuli were added. The wavelengths were set to 540 and 580 nm for excitation and emission respectively (Wollheim & Pozzan, 1984).

Statistical analysis

Results are given as means ± S.E.M. and statistical analyses were done by Student's t test for unpaired data.

Materials

The sources of materials used were as described previously (Wollheim & Pozzan, 1984), except for arginine-vasopressin (AVP) which was generously supplied by Dr. P. Melin, Ferring Pharmaceuticals, Malmö, Sweden; 1-oleoyl-2-acetylglycerol, PMA, carbachol and 1,2-dioleoyl-*rac*-glycerol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; t.l.c. plates of silica gel 60 were from Merck, Darmstadt, Germany; and [2-^{3}H]glycerol, [γ-^{32}P]ATP and cyclic AMP kits were from Amersham International, Amersham, Bucks, U.K. [^{3}H]Phorbol dibutyrate and ^{125}I-Protein A were from New England Nuclear, Boston, MA, U.S.A., and fura-2/acetoxymethyl ester and bisoxonol were from Molecular Probes, Eugene, OR, U.S.A. The polyclonal antibody against PKC was kindly provided by Dr. D. Fabbro, University of Basel, Basel, Switzerland. All other chemicals were of analytical grade.

RESULTS

PKC in 24 h-PMA-treated cells

In control cells about 92% of total PKC activity and of phorbol ester binding was found in the cytosol and 8% was present in the membranes (Table 1). The PKC of RINm5F cells was found by Western blotting using polyclonal antibodies directed against synthetic peptides of α, β and γ PKC isoenzymes to be of the α and β types (results not shown). After 24 h of treatment of cells with PMA (100 nM), PKC activity and phorbol ester binding were no longer detectable in either the cytosolic or the membrane fraction (Table 1). Using the detection limits indicated in Table 1, total PKC activity and phorbol ester binding in PMA-treated cells were less than 1% and 4% of control values respectively. The disappearance of PKC was also confirmed by immunoblotting (see Table 1) with a polyclonal antibody against PKC which cross-reacts with all isoforms of the enzyme. Total residual PKC immunoreactivity was less than 4% in PKC-deficient cells. Thus PMA treatment decreased PKC to undetectable levels, as demonstrated by three different means.

Effect of 24 h of PMA treatment on insulin secretion from perifused cells

The insulin release in response to various secretagogues was tested during perifusion of cells. Acute addition of PMA (100 nM) caused a gradual increase in insulin secretion to up to 4-fold that in control cells (Fig. 1*a*). There was only partial return towards basal levels upon cessation of stimulation. As expected, PMA failed to elicit any increase in secretion rate in cells whose PKC activity had been down-regulated (Fig. 1*a* and Table 2). In contrast, OAG (5 μg/ml), which only caused a 2-fold increase in insulin secretion in control cells, was still capable of stimulating secretion from cells treated for 24 h with PMA. OAG was even more efficient in causing insulin secretion in the PKC-down-regulated cells compared with its effects in paired control cells (Fig. 1*b* and Table 2).

Next we examined the action of two receptor agonists, AVP and carbachol, which activate PKC in these cells. In control cells the enzyme activity was 1044 ± 42 and 70 ± 5 units/mg of protein ($n = 3$) in cytosol and membranes respectively. After 2 min of incubation with 1 μM-AVP, the corresponding values were 988 ± 44 and 105 ± 6 units/mg of protein ($n = 3$). This 50% increase in membrane-associated PKC activity caused by AVP is comparable with the results obtained under the same conditions with 100 μM-carbachol (Wollheim & Regazzi, 1990). Both AVP (1 μM) and carbachol (100 μM) induced a monophasic, about 3-fold, increase in insulin release from control cells (Figs. 2*a* and

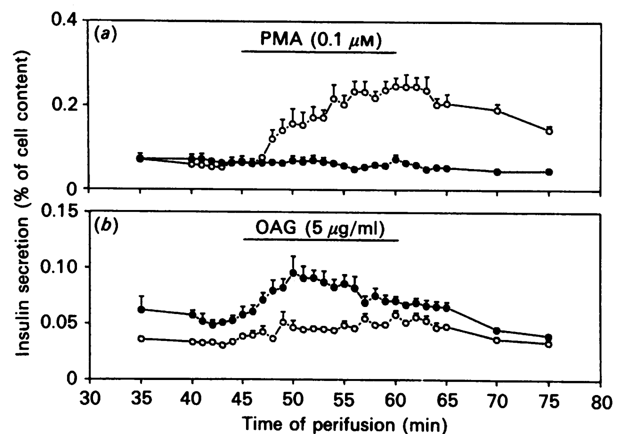

Fig. 1. Effects of PMA and OAG on insulin release from control and PKC-deficient RINm5F cells

Cells were perifused for 45 min before addition of 0.1 μM-PMA (*a*) or 5 μg of OAG/ml (*b*) for 15 min. Values are means ± S.E.M. ○, Control cells ($n = 6$); ●, 24-h-PMA-treated cells ($n = 6$).

Table 1. Effect of 24 h of PMA (100 nM) treatment on PKC activity, phorbol ester binding and PKC immunoreactivity in RINm5F cells

PKC activity, [^{3}H]phorbol dibutyrate binding and immunoblotting with affinity-purified antibodies against PKC were performed in cytosol and crude membrane fractions of RINm5F cells as described in the Experimental section. The immunoblot was quantified by densitometric analysis of the film. The results of the phorbol ester binding are given as means ± S.E.M. from three independent experiments. Note that the measurements of PKC activity, phorbol ester binding and PKC immunoreactivity were carried out in different experiments, i.e. Expt. 1 was different in each case.

		PKC activity (units/mg of protein)		Phorbol ester binding (fmol/mg of protein)		PKC immunoreactivity (arbitrary units)	
		Cytosol	Membranes	Cytosol	Membranes	Cytosol	Membranes
Expt. 1	Control	1349	59	1542 ± 251	133 ± 30	439	94
	Treated	5	< 5	< 30	< 30	< 10	< 10
Expt. 2	Control	1689	197				
	Treated	< 5	< 5				

Table 2. Effect of various secretagogues on insulin secretion from perifused control and PKC-deficient RINm5F cells

Cells were perifused for 45 min before addition of stimulus for 15 min. Values are means ± S.E.M. (numbers of observations in parentheses) of insulin release during a 15 min stimulation after subtraction of prestimulatory rates, which were 0.27 ± 0.02 % (n = 45) and 0.37 ± 0.04 % (n = 44) of cell content/5 min in control and PMA-treated cells respectively. * $P < 0.05$ and ** $P < 0.001$ compared with control. IBMX, 3-isobutyl-1-methylxanthine.

	Incremental insulin secretion (% of cell content)	
Stimulus	Control	24-h PMA-treated
Vasopressin (1 µM)	1.07 ± 0.19 (9)	5.88 ± 0.56** (9)
Carbachol (100 µM)	0.56 ± 0.11 (6)	2.62 ± 0.69* (6)
D-Glyceraldehyde (10 mM)	3.75 ± 0.46 (4)	8.05 ± 1.36* (3)
Alanine (10 mM)	1.96 ± 0.22 (8)	3.92 ± 0.21* (8)
OAG (5 µg/ml)	0.20 ± 0.05 (6)	0.41 ± 0.06* (6)
PMA (100 nM)	1.61 ± 0.31 (6)	0.03 ± 0.01** (6)
Forskolin (5 µM) + IBMX (0.5 mM)	0.11 ± 0.04 (6)	0.18 ± 0.04 (6)

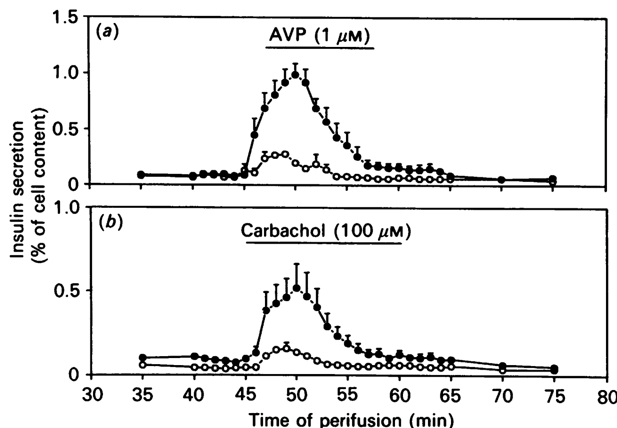

Fig. 2. Effects of AVP and carbachol on insulin release from control and 24-h-PMA-treated RINm5F cells

Cells were perifused for 45 min before addition of 1 µM-AVP (n = 9) (a) or 100 µM-carbachol (n = 6) (b) for 15 min. Values are means ± S.E.M. ○, Control cells; ●, 24-h-PMA-treated cells.

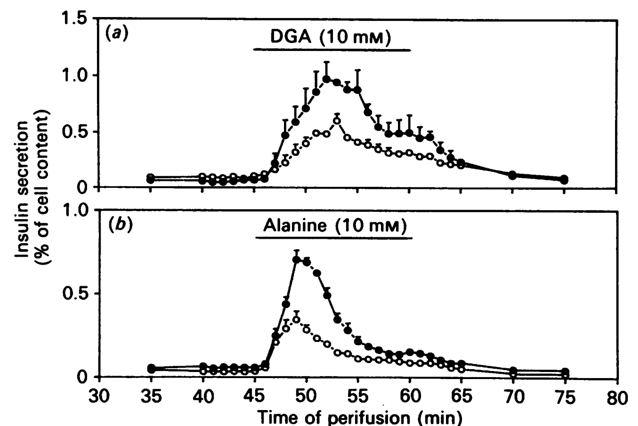

Fig. 3. Effects of D-glyceraldehyde (DGA) and alanine on insulin release from control and PKC-deficient RINm5F cells

Cells were perifused for 45 min before addition of 10 mM-D-glyceraldehyde (a) or 10 mM-alanine (b) for 15 min. Values are means ± S.E.M. ○, Control cells (a, n = 4; b, n = 8); ●, 24-h-PMA-treated cells (a, n = 3; b, n = 8).

2b). The stimulation was transient and the secretion rate returned to basal before the discontinuation of the stimuli. After a 24 h PMA treatment, the insulin release induced by both agents was markedly potentiated. The peaks of secretion rates were increased by 3- and 2.5-fold compared with controls with AVP and carbachol respectively. The secretion induced by both agents was also of longer duration in PKC-deficient cells (Figs. 2a and 2b). In both cases, integrated incremental insulin secretion was five times greater in PKC-deficient cells (Table 2).

To determine whether nutrient-stimulated insulin release is also affected by long-term PMA treatment, we examined the effects of the metabolizable substrates D-glyceraldehyde and alanine, which have previously been shown to promote secretion from these cells (Wollheim & Pozzan, 1984). D-Glyceraldehyde (10 mM) and alanine (10 mM) caused 6-fold increases in peak secretion rates in control cells. The secretion rates returned to basal values only when the stimuli were stopped. Again, stimulated insulin secretion was clearly enhanced in long-term-PMA-treated cells, resulting in a doubling of total insulin output (Fig. 3 and Table 2).

Since the secretagogues used so far have been shown to raise $[Ca^{2+}]_i$ levels in RINm5F cells (Wollheim & Pozzan, 1984; Wollheim & Biden, 1986; Li *et al.*, 1988; Martin *et al.*, 1989), we next tested whether an increase in cellular cyclic AMP levels, a condition not associated with a rise of $[Ca^{2+}]_i$ in these cells (Wollheim *et al.*, 1984), also leads to enhanced secretion from PKC-down-regulated cells. Forskolin (5 µM) plus 0.5 mM-3-isobutyl-1-methylxanthine elicited a small increase in insulin release from control cells. After 24 h of PMA treatment, insulin release induced by these agents showed a tendency to be augmented, but the difference was not statistically significant (Table 2). Thus insulin secretion from PKC-deficient cells was enhanced in response to agents utilizing Ca^{2+} rather than cyclic AMP as intracellular messenger.

Effect of 24 h of PMA treatment on cyclic AMP production in RINm5F cells

Since agents which increase cyclic AMP levels are known to stimulate insulin secretion (Prentki & Matschinsky, 1987), we examined whether PKC down-regulation enhanced cellular cyclic AMP levels. When cells were incubated for 3 min under basal and stimulated conditions, 24 h of PMA treatment caused a 26 % decrease in basal cyclic AMP levels. Forskolin elicited a 3-fold increase in cyclic AMP content in both preparations. AVP did not affect the generation of cyclic AMP in either control or PMA-treated cells (Table 3). The enhanced insulin secretion from PKC-down-regulated cells is therefore not associated with an increased generation of cyclic AMP.

$[Ca^{2+}]_i$ in control and PMA-treated cells

An elevation in $[Ca^{2+}]_i$ is one of the most important determinants of insulin secretion (Wollheim & Biden, 1986; Prentki & Matschinsky, 1987). Therefore changes in $[Ca^{2+}]_i$ in response to the different secretagogues were assessed in PMA-treated cells.

Fig. 4(a) shows that 1 µM-AVP induced a biphasic increase in $[Ca^{2+}]_i$ in suspensions of control cells. The rise comprised a big initial transient followed by a small sustained elevation. The action of AVP was inhibited by adding $d(CH_2)_5Tyr(Me)AVP$, a V_1 receptor antagonist which returned $[Ca^{2+}]_i$ to basal levels (Figs. 4a and 4b). Short-term (5 min) exposure of RINm5F cells to PMA (10–100 nM) completely abolished the $[Ca^{2+}]_i$ rise due to AVP (not shown). In contrast, after a 24 h PMA treatment the

Table 3. Effects of AVP and forskolin on cyclic AMP content of control and 24-h-PMA-treated RINm5F cells

After a 10 min preincubation, the cells were incubated with or without test substances for 3 min at 37 °C. Cyclic AMP was measured with a radioimmunoassay kit. Values are means ± S.E.M. of the numbers of observations given in parentheses. * $P < 0.05$ compared with control.

Conditions	Cyclic AMP (pmol/10^6 cells)	
	Control	24-h PMA-treated
Basal	1.48 ± 0.06 (9)	1.09 ± 0.04* (9)
AVP (1 μM)	1.44 ± 0.08 (9)	1.17 ± 0.07* (9)
Forskolin (8 μM)	4.00 ± 0.024 (9)	3.34 ± 0.19* (10)

first $[Ca^{2+}]_i$ peak due to AVP became larger and lasted longer. The duration of the first $[Ca^{2+}]_i$ peak was significantly longer in PKC-deficient cells (Fig. 4*b* and Table 4). However, neither the basal $[Ca^{2+}]_i$ levels nor the amplitudes of the first peak and the second phase differed between control and 24-h-PMA-treated cells (Table 4).

To analyse in more detail the kinetics of the $[Ca^{2+}]_i$, we also performed the experiments at the single-cell level. Fig. 5 illustrates the $[Ca^{2+}]_i$ changes induced by 0.1 μM-AVP in single RINm5F cells. In the control cells the $[Ca^{2+}]_i$ response was characterized by a large initial transient followed by small $[Ca^{2+}]_i$ oscillations. In the PMA-treated cells the initial $[Ca^{2+}]_i$ rise lasted longer and the subsequent $[Ca^{2+}]_i$ oscillations became confluent. These results confirm those seen in cell suspensions.

Comparison of the effects of other secretagogues on $[Ca^{2+}]_i$ in

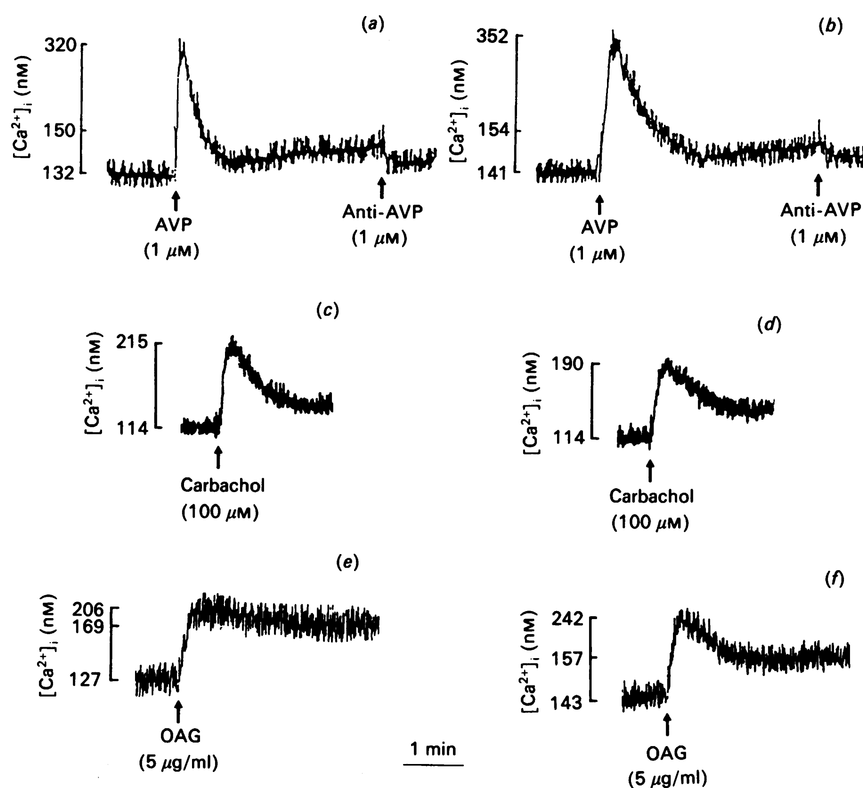

Fig. 4. Effects of AVP, carbachol and OAG on $[Ca^{2+}]_i$ in control and PKC-deficient RINm5F cell suspensions

Cells were loaded with 1 μM-fura-2/acetoxymethyl ester for 30 min at 37 °C. Traces are representative of at least four observations in each case. For means values, see Table 3. (*a*), (*c*) and (*e*), Control; (*b*), (*d*) and (*f*), 24-h-PMA-treated. Anti-AVP, $d(CH_2)_5Tyr(Me)AVP$.

Table 4. Effects of various secretagogues on $[Ca^{2+}]_i$ in control and PKC-deficient RINm5F cells

Cells were loaded with 1 μM-fura 2/acetoxymethyl ester for 30 min at 37 °C and $[Ca^{2+}]_i$ was measured as described in the Experimental section. Values are means ± S.E.M. * $P < 0.001$ compared with control. † Duration of initial peak. DGA, D-glyceraldehyde.

Stimulus	Control					24-h PMA-treated				
	$[Ca^{2+}]_i$ (nM)					$[Ca^{2+}]_i$ (nM)				
	Basal	Peak	Sustained	Duration	*n*	Basal	Peak	Sustained	Duration	*n*
Vasopressin (1 μM)	127 ± 4	287 ± 11	140 ± 4	47 ± 3 s†	9	118 ± 2	257 ± 21	133 ± 2	70 ± 2 s*†	7
Carbachol (100 μM)	130 ± 10	228 ± 16	–	68 ± 9 s	4	117 ± 7	209 ± 14	–	76 ± 7 s	4
DGA (10 mM)	132 ± 7	188 ± 9	180 ± 10	> 5 min	4	129 ± 7	210 ± 31	184 ± 16	> 5 min	4
Alanine (10 mM)	125 ± 3	196 ± 13	162 ± 9	> 3 min	4	129 ± 6	186 ± 19	157 ± 13	> 3 min	4
OAG (5 μg/ml)	139 ± 7	218 ± 22	166 ± 6	> 3 min	5	136 ± 7	220 ± 19	162 ± 5	> 3 min	6

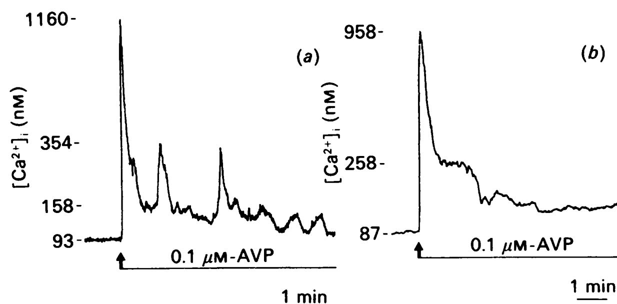

Fig. 5. Effect of AVP on $[Ca^{2+}]_i$ in single RINm5F cells

Cell monolayers were loaded with 1 μM-fura-2/acetoxymethyl ester for 20 min and fluorescence was measured by dual-wavelength microfluorometry. The traces represent the calibrated ratio of fluorescence after excitation at 340 and 380 nm. For details, see Pralong *et al.*, (1990). Traces are representative of six observations (*a*) and five in (*b*). (*a*) Control; (*b*) PMA-treated.

suspensions of control and PKC-down-regulated cells did not reveal similar changes as those observed with AVP. The $[Ca^{2+}]_i$ rise due to carbachol is shown in Figs. 4(*c*) and 4(*d*), and pooled results are given in Table 4. As reported previously for 1,2-didecanoylglycerol (Wollheim *et al.*, 1988), OAG also raised $[Ca^{2+}]_i$ in RINm5F cells. The response was unaltered by long-term PMA treatment (Figs. 4*e*, and 4*f* and Table 4). Likewise, the $[Ca^{2+}]_i$ rises elicited by D-glyceraldehyde and alanine, which were similar to those published previously (Wollheim & Pozzan, 1984), were not different after PKC down-regulation (Table 4).

DAG production due to AVP in the PKC-deficient cells

AVP is known to activate PKC via V_1 receptors (Stassen *et al.*, 1989). The activity of this enzyme was monitored by measuring the generation of DAG. AVP (1 μM) increased DAG production in control cells, peaking at 2 min and thereafter declining, but reaching a level still higher than basal at 15 min (Fig. 6). When the cells were treated with PMA for 24 h, the DAG accumulation induced by AVP was much faster and significantly higher than that of control cells at the early time points. At 2 min and later the alteration of DAG production was less marked. As expected, when cells were pretreated with PMA acutely (2 min), the AVP-induced increase in DAG accumulation was completely inhibited (97.6±3.2% of basal, $n = 4$).

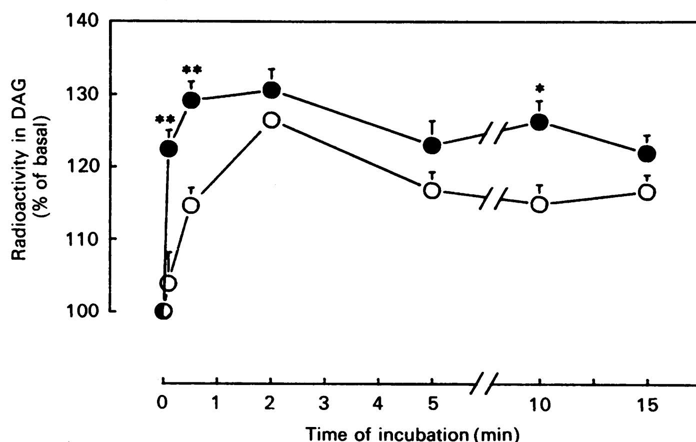

Fig. 6. Effect of AVP on DAG accumulation in control and PKC-down-regulated RINm5F cells

Cells were labelled with [³H]glycerol for 48 h and DAG was measured as indicated in the Experimental section. Values are means±S.E.M. for 6–9 observations. * $P < 0.05$ and ** $P < 0.01$ compared with control. ○, Control cells; ●, 24-h-PMA-treated cells.

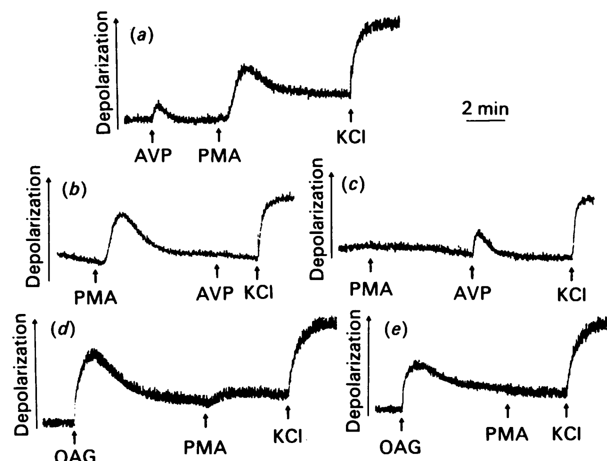

Fig. 7. Effects of AVP, PMA and OAG on membrane potential in control and PKC-deficient RINm5F cells

Membrane potential was monitored with the fluorescent probe bisoxonol (100 nM). (*a*), (*b*) and (*d*), Control; (*c*) and (*e*), 24-h-PMA-treated. Traces are representative of at least four observations in each case. Concentrations used were 1 μM-AVP, 100 nM-PMA, 24 mM-KCl and 5 μg of OAG/ml.

Membrane potential in short- and long-term PMA-treated cells

Activators of PKC such as PMA and 1,2-didecanoylglycerol have been shown to depolarize RINm5F cells by closing ATP-sensitive K^+ channels (Wollheim *et al.*, 1988). To investigate whether the increased generation of DAG is associated with alteration of membrane potential, this parameter was monitored using the fluorescent probe bisoxonol. First, the effects of short-term exposure of the cells to PMA on AVP-induced membrane depolarization were examined. Fig. 7(*a*) shows that AVP (1 μM) caused a transient depolarization of the cells to a level 25% of that seen after an increase of K^+ from 6 to 30 mM. PMA (100 nM) caused a larger depolarization and completely blocked the response to AVP when the latter was added when the membrane potential had partially repolarized (Fig. 7*b*). After 24 h of pretreatment of the cells with PMA, addition of the phorbol ester no longer depolarized the cells, whereas AVP promoted membrane depolarization as in control cells (Fig. 7*c*). The membrane depolarization induced by D-glyceraldehyde and alanine was not affected by PKC depletion (results not shown).

Finally, it was interesting to test the effects of OAG on membrane potential in view of the preserved action of the DAG on insulin secretion and $[Ca^{2+}]_i$ rise in 24 h-PMA-treated cells (Fig. 1*b*, Table 2 and Table 4). OAG (5 μg/ml) depolarized the membrane to a similar extent in both control (Fig. 7*d*) and PKC-down-regulated (Fig. 7*e*) cells.

Effect of 24 h of PMA treatment on insulin release from permeabilized cells

In order to investigate whether PKC down-regulation causes a general sensitization of the mechanism of exocytosis, insulin secretion was also measured in electrically permeabilized cells. As published previously (Vallar *et al.*, 1987), an increase in the Ca^{2+} concentration from 0.1 to 2 and 10 μM caused a dose-related stimulation of insulin secretion (Fig. 8). The effects of AVP were examined at 2 μM-Ca^{2+}, the concentration which is half-maximal for stimulation of secretion (Vallar *et al.*, 1987). AVP (10 μM) increased Ca^{2+}-stimulated insulin release by 18 and 28% relative

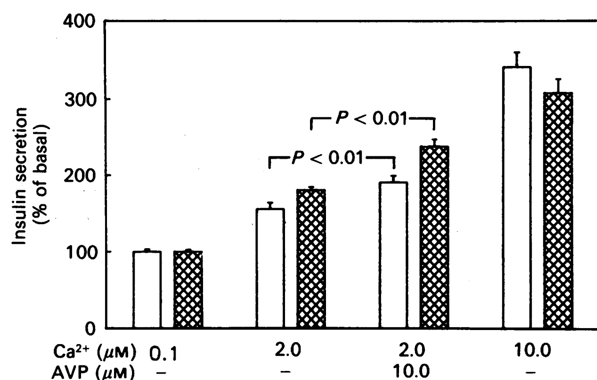

Fig. 8. Ca^{2+} and AVP-stimulated insulin secretion from electrically permeabilized RINm5F cells

Cells were permeabilized and incubated as described in the Experimental section. Ca^{2+} concentrations in the incubation medium were buffered with Ca^{2+}/EGTA and measured with a Ca^{2+}-selective electrode. Values are mean ± S.E.M. for 15 observations from three independent experiments. Basal insulin release at 0.1 μM-Ca^{2+} was 20.9 ± 1.6 (n = 15) in controls and 18.3 ± 1.7 (n = 15) ng/5 min per 10^6 cells in PKC-down-regulated cells. □, Control cells; ▩, 24-h-PMA-treated cells.

to the rate at 2 μM-Ca^{2+} in control and PMA-treated cells respectively. The secretion in response both to 2 μM-Ca^{2+} alone and to AVP was significantly, albeit only slightly, higher in PKC-deficient cells ($P < 0.05$). In contrast, insulin secretion was not different between control and 24 h-PMA-treated cells under basal (legend to Fig. 8) and at maximally stimulatory (Fig. 8) Ca^{2+} conditions. This suggests that the large potentiation of insulin secretion in intact cells cannot be explained by sensitization of the secretory process to Ca^{2+} and AVP in PKC-down-regulated cells.

DISCUSSION

For the unequivocal interpretation of results from PKC down-regulation experiments, it was important to demonstrate that functional PKC is no longer detectable after long-term phorbol ester treatment. The RINm5F cells showed such marked down-regulation, as demonstrated in cytosol as well as in membranes by enzymic activity, phorbol ester binding and immunoblotting. In pancreatic islets (Arkhammar *et al.*, 1989; Persaud *et al.*, 1989*b*) and in HIT cells (Hughes *et al.*, 1990), treatment with twice the concentration of PMA (200 nM) used here only resulted in a partial depletion of cytosolic PKC and the activity of the membrane-associated enzyme was retained. While these studies found glucose-induced insulin secretion to be unchanged (Arkhammar *et al.*, 1989; Persaud *et al.*, 1989*b*) or slightly decreased (Hughes *et al.*, 1990), muscarinic agonist-mediated potentiation of glucose-stimulated insulin release was diminished (Persaud *et al.*, 1989*b*) or abolished (Hughes *et al.*, 1990). These results contrast markedly with the present findings that in PMA-treated RINm5F cells, the secretory response to a wide variety of stimuli was enhanced after PKC down-regulation. This was true for D-glyceraldehyde and alanine which, like glucose, primarily cause membrane depolarization and Ca^{2+} influx via voltage-dependent Ca^{2+} channels (Wollheim & Pozzan, 1984), as well as for carbachol and vasopressin, which primarily mobilize Ca^{2+} from intracellular stores (Wollheim & Biden, 1986; Li *et al.*, 1988; Martin *et al.*, 1989). In two other islet studies, long-term treatment with PMA enhanced glucose-induced insulin secretion (Metz, 1988; Thams *et al.*, 1990). In the latter report, glucose-induced insulin secretion was enhanced during the first phase and decreased during the second phase. Similar results were seen with carbachol.

The most straightforward explanation for the enhanced secretory response is the elimination of an inhibitory influence of PKC in control cells. Enhanced secretion would thus only occur after complete, but not partial, depletion of functional PKC. Such feedback inhibition would then occur not only with agents known to activate PKC such as carbachol (Yamatani *et al.*, 1988; Persaud *et al.*, 1989*a*; Arkhammar *et al.*, 1989; Regazzi *et al.*, 1990) and AVP, but also with the metabolizable substrates such as D-glyceraldehyde and alanine. However, secretion was not significantly enhanced on stimulation with forskolin, which activates adenylate cyclase but does not raise $[Ca^{2+}]_i$ in RINm5F cells (Wollheim *et al.*, 1984). To test whether a general sensitization of the secretory process had occurred, we also examined insulin secretion from electrically permeabilized cells. Although there was a tendency for higher secretion rates at 2 μM-Ca^{2+}, the effects were small and were not observed at maximal Ca^{2+} concentrations. It was of interest that the receptor agonist AVP is still capable of eliciting a small increase of insulin secretion from the permeabilized cells and that this effect is not dependent on functional PKC. The secretory machinery of PKC-down-regulated cells does not, therefore, display a marked increase in the sensitivity to Ca^{2+} and to activation of phospholipase C.

The results with permeabilized cells suggest that soluble messenger molecules could be mediating the enhanced secretory response following long-term PMA treatment. In intact cells cyclic AMP levels were somewhat lower in PKC-down-regulated cells in the face of comparable increases elicited by forskolin. As expected, AVP did not enhance cyclic AMP levels in either cell preparation, confirming that the agonist only activates V_1 receptors in RINm5F cells (Li *et al.*, 1988). Therefore increased cyclic AMP production cannot explain the enhanced insulin secretion in PKC-down-regulated cells.

With respect to $[Ca^{2+}]_i$, only the AVP response was clearly enhanced after long-term PMA treatment. Thus the response to carbachol, the activation of phospholipase C, or the effects of nutrients opening Ca^{2+} channels did not reveal any detectable differences between control and PKC-down-regulated cells. It is therefore unlikely that changes in cellular Ca^{2+} handling underlie the augmented insulin secretion. This conclusion is at variance with the results from other cell types, where different receptor agonists caused amplified inositol trisphosphate production (Brown *et al.*, 1987; Hepler *et al.*, 1988; Vicentini *et al.*, 1988; Stassen *et al.*, 1989; Pfeilschifter *et al.*, 1989) and $[Ca^{2+}]_i$ rises (Pandiella *et al.*, 1987; Stassen *et al.*, 1989; Pfeilschifter *et al.*, 1989) after PKC down-regulation. In our study only AVP showed enhanced phospholipase C activation, as demonstrated by increased DAG production and changes in the $[Ca^{2+}]_i$ profile. The latter was not observed with carbachol. The difference between the two Ca^{2+}-mobilizing agonists could be due to different sensitivity to the inhibitory influence of PKC exerted at the level of either the receptor or the GTP-binding protein coupling the receptor to phospholipase C. It was interesting that AVP did not cause $[Ca^{2+}]_i$ oscillations in PKC-deficient cells. This may indicate a role for PKC in the generation of $[Ca^{2+}]_i$ oscillations, as suggested previously for the effects of AVP and other Ca^{2+}-mobilizing agonists in hepatocytes (Cobbold *et al.*, 1989) and bradykinin in artery endothelial cells (Sage *et al.*, 1989).

The finding that the synthetic DAG analogue, OAG, also promoted increased insulin secretion and still depolarized the cells after PKC down-regulation is of dual interest. On the one hand, it provides further support for the conclusion that the main site exhibiting increased sensitivity to the various secretagogues is located distal to signal generation by phospholipase C and the rise in $[Ca^{2+}]_i$. On the other hand, this is another example

of an effect of OAG not mediated by PKC. It has previously been reported that OAG and other synthetic DAGs stimulate phospholipase A_2 (Burch, 1988), inhibit lysophosphatide acyltransferase (Goppelt-Strübe *et al.*, 1987) and stimulate sphingomyelin hydrolysis (Kolesnick, 1987) in a PKC-independent manner. In view of this, it is possible that arachidonic acid or one of its numerous metabolites could be involved in the enhanced efficacy of the secretagogues in the PMA-treated cells. These metabolites would not accumulate in permeabilized cells. However, the difference in the secretory responses between intact and permeabilized cells could be explained, not by the loss of putative soluble messenger molecules, but by a difference in energized state. Alterations of given metabolic pathways in PKC-down-regulated cells would presumably not be operative in permeabilized cells, whose main source of energy is exogenous ATP. The exaggerated secretory response observed in PKC-deficient cells could thus be due to an alteration in the gene expression of enzymes in metabolic pathways, the impact of which would be lost after cell permeabilization. Further studies are required for the elucidation of the adaptive changes in the long-term-PMA-treated cells. The straightforward conclusion that PKC is not mediating the action on insulin secretion of the muscarinic agonist carbachol and of AVP acting on V_1 receptors is premature. It is likely that other protein kinases regulated by Ca^{2+} can substitute for PKC in such treated cells. This does not seem to apply to all protein kinases, since agents which raise cyclic AMP levels did not cause augmented insulin secretion in PKC-deficient cells. An alternative, albeit unlikely, explanation for the retained efficacy of carbachol, AVP and OAG is that some PKC species escape down-regulation and detection by the methods used here.

Using RINm5F cells, we have previously proposed that PKC could mediate in part the action of D-glyceraldehyde on insulin secretion by promoting closure of ATP-sensitive K^+ channels, in turn leading to membrane depolarization (Wollheim *et al.*, 1988). The retained efficacy of D-glyceraldehyde in PKC-deficient cells suggests either that PKC is not involved in the signal transduction caused by carbohydrates or that adaptive changes such as those mentioned above compensate for the loss of PKC activity. As discussed elsewhere (Wollheim & Regazzi, 1990; Regazzi *et al.*, 1990), it has proved exceedingly difficult to establish a role for PKC in carbohydrate-induced insulin secretion.

It is well established that activation of PKC interferes with the coupling of receptors to phospholipase C (Nishizuka, 1988; Kikkawa *et al.*, 1989). This was also demonstrated here for AVP, as short-term exposure of the cells to PMA blocked membrane depolarization, DAG production and the Ca^{2+} rise in response to the agonist. Since the potentiation of insulin secretion following long-term treatment with PMA is seen with agonists activating phospholipase C as well as with depolarizing agents, it is concluded that this effect is not restricted to the mechanism coupling receptors to their effector systems.

In conclusion, the present work extends the notion of feedback control by PKC of signal transduction via receptors activating phospholipase C to a more general inhibitory influence on insulin release induced by different secretagogues.

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