Phorbol ester inhibition of the hormone-stimulated phosphoinositide cycle in WRK-1 cells

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WRK-1 rat mammary tumour cells respond to vasopressin with increased accumulation of inositol phosphates as well as increased precursor incorporation into phosphatidylinositol. The phorbol ester, phorbol 13-myristate 12-acetate (PMA) inhibits by 80% both inositol phosphate accumulation and increased precursor incorporation. This inhibition is much less evident at early times (2 min) than at later times (25 min). The vasopressin-induced rise in cytosolic free Ca^{2+} is inhibited in a similar manner. Oleoylacetyl glycerol is inactive with respect to inhibition of vasopressin-induced increases in incorporation of ^{32}P into phosphoinositides. PMA has no effect on vasopressin binding at saturating concentrations of the hormone and does not affect the binding affinity.

INTRODUCTION

The phorbol ester tumour promoters have been reported to activate protein kinase C in a variety of systems (Nishizuka, 1984), as well as to synergize with Ca^{2+} ionophores in mimicking the activities of a number of Ca^{2+} -mobilizing hormones (Yamanishi *et al.*, 1983; Culty *et al.*, 1984). On the basis of these findings it has been suggested that tumour-promoter action is mediated by stimulation of protein kinase C, the activation of which constitutes one arm of the phosphoinositide pathway. Paradoxically, however, it has also been reported that phorbol esters inhibit both agonist-induced increases in incorporation of ^{32}P into phosphatidylinositol (Sahai *et al.*, 1982; Smith *et al.*, 1983) as well as accumulation of inositol phosphates (Labarca *et al.*, 1984). We report here that the same situation applies to vasopressin-induced phosphoinositide metabolism in WRK-1 cells.

MATERIALS AND METHODS

Tissue-culture media, sera, trypsin and Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) were supplied by Gibco. Phosphate-free medium was from Flow Laboratories. Insulin was purchased from Eli Lilly Co. Vasopressin was from Calbiochem. ^{32}P (^{32}P) P_i (carrier-free) was purchased from ICN. *myo*- ^3H inositol was from New England Nuclear. Phospholipid standards were from Sigma. Quin 2 AM was from Sigma. Thin-layer plates (IB2-F) were from Baker and organic solvents were purchased from Fisher. Aquasol II was from New England Nuclear. Synthetic OAG was purchased from Serdary.

Cell culture

WRK-1 cells were established from a dimethylbenz[*a*]-anthracene-induced rat mammary tumour as previously described (Kidwell *et al.*, 1978; Monaco *et al.*, 1978).

Growth characteristics and responses to neurohypophyseal hormones have also been described (Monaco & Lippman, 1982; Monaco & Woods, 1983). In brief, cells were grown in monolayer cultures in minimal essential medium containing Earle's salts and supplemented with penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), Fungizone (0.25 $\mu\text{g}/\text{ml}$), calf serum (5%, v/v) and rat serum (2%, v/v). For experiments, cells were harvested with a solution of trypsin (0.05%) and EDTA (0.02%) in 0.9% NaCl and replicately plated into 35 mm-diameter plastic dishes. Details for each experiment appear in the Figure legends. Vasopressin was routinely used at a concentration of 100 nM. PMA was solubilized in DMSO before addition to the cultures.

Measurement of ^{32}P incorporation into lipids

After incubation of cells with ^{32}P radioactive lipids were extracted and quantified as previously described (Monaco & Woods, 1983).

Measurement of ^3H inositol phosphate accumulation

Cells were plated and grown in ^3H inositol (10 $\mu\text{Ci}/\text{ml}$). When confluent, cells were harvested and resuspended in serum-free minimal essential medium containing 10 mM-LiCl. This concentration of Li^+ did not effect basal incorporation of precursors into lipids or cell viability. Cells were then incubated at 37 °C for 2 or 25 min with the additions indicated.

All samples contained 0.2% DMSO, the solvent used as vehicle for PMA; DMSO at this concentration has no observable effect on WRK-1 cells. At the end of the incubation period, cells were pelleted by centrifugation and extracted with chloroform/methanol/0.1 M-HCl (8:4:3, by vol.). The aqueous layer was dried down *in vacuo* and the inositol phosphates separated as previously described (Downes & Michell, 1981).

Abbreviations used: PMA, phorbol 13-myristate 12-acetate ('TPA'); OAG, oleoylacetyl glycerol; DMSO, dimethyl sulphoxide.

Measurement of cytosolic free Ca^{2+}

Cytosolic free Ca^{2+} concentrations were measured using quin 2 AM as a fluorescent indicator as previously described (Gershengorn & Thaw, 1983). In brief, 15 μM -quin 2 AM was incubated with cells for 20 min, followed by three washes with Hanks balanced salt solution. Minimum and maximum fluorescence values for quin 2 fluorescence were calculated with EGTA and Triton X-100 respectively.

Measurement of [^3H]vasopressin binding

Cells were harvested with a solution of EDTA (1 mM) in phosphate-buffered saline and resuspended [(1–3) $\times 10^6$ cells/ml] in 12 mm $\times$ 75 mm plastic tubes in serum-free minimal essential medium containing insulin (2 μM), Tricine (25 mM), bovine serum albumin (10 mg/ml) and bacitracin (75 units/ml). [^3H]Vasopressin (0.5–50 nM), plus or minus 1000-fold excess of unlabelled vasopressin) was added to each tube. Incubations were carried out for 30 min at 22 °C. Cells were then rapidly washed thrice with ice-cold phosphate-buffered saline, resuspended in 0.5 ml of phosphate-buffered saline and transferred to a scintillation vial for determination of radioactivity.

Measurement of [^{32}P]P_i incorporation into ATP

The aqueous phase remaining after the extraction of the cells with chloroform/methanol/0.1 M-HCl was chromatographed on silica-gel G in 0.8 M-LiCl. Nucleotide standards were detected with u.v. light.

RESULTS

Effect of PMA on vasopressin-induced [^{32}P]P_i incorporation into phosphatidylinositol

Fig. 1 illustrates the effect of PMA at 10 and 100 ng/ml on vasopressin-stimulated incorporation of [^{32}P]P_i into

Fig. 1. Effect of PMA on vasopressin-induced increases in [^{32}P]P_i incorporation into phosphatidylinositol (PI)

Cells were treated simultaneously with PMA, vasopressin (VP, 100 nM) and [^{32}P]P_i. After a 1 h incubation, cells were harvested and the lipids extracted and analysed as described in the Materials and methods section. Values represent the mean of triplicate determinations $\pm$ 1 S.D.

phosphatidylinositol. Whereas PMA at 100 ng/ml was slightly stimulatory by itself (in the absence of hormone), concentrations of as little as 10 ng/ml profoundly inhibited hormone-stimulated incorporation. In a series of six experiments, inhibition by PMA at 100 ng/ml averaged 88%.

Effect of TPA on [^{32}P]P_i incorporation into ATP, phosphatidylcholine and phosphatidylethanolamine

To determine whether or not PMA was having any general inhibitory effect on [^{32}P]P_i incorporation which might account for the inhibition of the vasopressin effect on phosphatidylinositol, [^{32}P]P_i incorporation into other phospholipids, as well as into ATP, was assessed. Table 1 details the results. Although PMA had little or no effect on the labelling of ATP, it significantly enhanced the incorporation of [^{32}P]P_i into phosphatidylcholine and phosphatidylethanolamine.

Effect of PMA on inositol phosphate accumulation

To determine if the attenuation of [^{32}P]P_i incorporation was the result of inhibition of the first step in hormonal alteration of phosphoinositide metabolism, breakdown of phosphatidylinositol 4,5-bisphosphate was monitored by measuring the accumulation of the inositol phosphate breakdown products. Table 2 details the results. PMA inhibited vasopressin-induced accumulation of all the inositol phosphates by 80% in this experiment. Since quin 2 and binding experiments (see below) were carried out at 22 °C, we also measured the inhibitory effect of PMA on inositol phosphate accumulation at this temperature. Identical results were obtained (Table 3).

Table 1. Effect of PMA on incorporation of [^{32}P]P_i into ATP, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in WRK-1 cells

Values shown are the averages for five separate experiments. The range is given in parentheses. *indicates a statistically significant change.

Metabolite	Incorporation (% of control)
ATP	121 (78–147)
PC	1004* (263–2348)
PE	322* (234–420)

Table 2. Effect of PMA on vasopressin-induced inositol phosphate accumulation in WRK-1 cells

Results are the means of triplicate determinations $\pm$ 1 S.D. Similar results were obtained in three separate experiments. Abbreviations: VP, vasopressin; IP, IP₂, IP₃, inositol mono-, bis- and tris-phosphate.

Addition	³² P radioactivity accumulated (c.p.m.)		
	IP	IP ₂	IP ₃
None	1927 $\pm$ 285	668 $\pm$ 87	544 $\pm$ 42
VP (100 nM)	16646 $\pm$ 1323	2756 $\pm$ 177	1653 $\pm$ 96
PMA (100 ng/ml)	1792 $\pm$ 363	503 $\pm$ 76	423 $\pm$ 30
VP + PMA	4582 $\pm$ 401	640 $\pm$ 77	502 $\pm$ 94

Table 3. Effect of PMA on vasopressin-induced inositol phosphate accumulation in WRK-1 cells at 22 °C

Results are the means of triplicate determinations $\pm$ 1 s.d.
Abbreviation used: VP, vasopressin.

Addition	Radioactivity in inositol phosphates (c.p.m.)
PMA (100 ng/ml)	3723 $\pm$ 311
VP (100 nM)	9939 $\pm$ 632
VP + TPA	5083 $\pm$ 481

Fig. 2. Effect of PMA on the vasopressin-induced rise in intracellular free Ca^{2+}

Cells previously harvested, suspended and loaded with quin 2 were incubated with (b) or without (a) PMA, (100 ng/ml) as indicated, followed by addition of vasopressin (VP, 100 nM) where indicated. The results shown are for a single experiment. Two other experiments gave similar results.

Effect of PMA on vasopressin-induced Ca^{2+} mobilization

The ability of vasopressin to increase the level of intracellular Ca^{2+} is shown in Fig. 2(a). Fig. 2(b) illustrates the effect of vasopressin after a 2 min preincubation with PMA. No attenuation of the hormone's effect is observed, and PMA alone has no effect. Vasopressin raised intracellular Ca^{2+} levels from 199 nM to 689 nM in the absence of PMA and from 156 nM to 783 nM in its presence. However, if cells are preincubated with PMA for 25 min before the addition of hormone, vasopressin-induced mobilization of Ca^{2+} is strongly inhibited (Fig. 3). In the absence of PMA, vasopressin increased free Ca^{2+} concentrations from 186 nM to 1897 nM, whereas after incubation with PMA for 25 min the vasopressin-stimulated value was only 373 nM.

Effect of PMA on inositol phosphate accumulation at early times

The fact that PMA did not block vasopressin-induced Ca^{2+} mobilization unless cells were preincubated for 25 min with PMA suggested that hormone-induced inositol phosphate accumulation might also be intact if PMA were only present for a short time. To test this, we measured inositol phosphates after 2 min of stimulation with vasopressin in the presence and absence of simultaneously added PMA. Table 4 illustrates the results of two separate experiments. The effect of PMA seen at this time is markedly less than that seen at 25 min.

Effect of OAG on the phosphoinositide cycle

The synthetic diacylglycerol analogue OAG has been shown to stimulate protein kinase C activity in whole cells (Kaibuchi *et al.*, 1983) and as such might be expected to have effects similar to those seen with PMA. We tested the ability of OAG to inhibit vasopressin-induced phosphoinositide metabolism. Table 5 illustrates the results. OAG at 100 $\mu\text{g}/\text{ml}$ was without a statistically significant effect.

Fig. 3. Effect of preincubation with PMA on the vasopressin-induced rise in intracellular free Ca^{2+}

Cells previously loaded with quin 2 were preincubated with PMA (100 ng/ml) for 25 min before the addition of vasopressin at zero time (broken line). The continuous line represents values for control cells. In each case, vasopressin (100 nM) was added at 75 s.

Table 4. Effect of PMA on vasopressin-induced inositol phosphate accumulation at early times

Results given are for two or three separate experiments. Each experiment was done in triplicate. Abbreviations: VP, vasopressin; IP etc., see Table 2.

Addition and time	Inhibition by TPA (%)		
	IP	IP ₂	IP ₃
PMA (100 ng/ml, 2 min) + VP (100 nM, 2 min)	0, 7, 8	16*, 0, 24*	23, 18*, 10
PMA (25 min) + VP (2 min)	49*, 29	67*, 55*	66*, 55*

* Statistically significant inhibition ($P = 0.05$).

Table 5. Effect of OAG on vasopressin-induced incorporation of [³²P]PI into phosphatidylinositol

Results are the means of triplicate determinations $\pm$ 1 S.D. Abbreviations: VP, vasopressin; PI, phosphatidylinositol.

Addition	[³² P]PI (c.p.m.)
None	2966 $\pm$ 804
VP (100 nM)	24869 $\pm$ 2763
PMA (100 ng/ml)	9997 $\pm$ 1404
OAG (100 μ g/ml)	2955 $\pm$ 298
VP + PMA	7313 $\pm$ 1087
VP + OAG	19012 $\pm$ 2008
PMA + OAG	5935 $\pm$ 514

Table 6. Effect of PMA on [³H]vasopressin binding to WRK-1 cells

Methods were as described in the text.

Addition	Sites/cell		K_d (nM)
	Expt. 1	Expt. 2	
None	13886	14870	1.02
PMA (200 ng/ml)	14736	20339	1.43

Effect of PMA on binding of [³H]vasopressin to WRK-1 cells

Table 6 details results of binding experiments carried out in the presence and absence of PMA. We have previously determined that the receptor on these cells is saturated between 20 and 50 nM-vasopressin (results not shown). A saturating dose was used to determine the number of sites per cell. In two separate experiments, PMA failed to lower the number of specific sites. A third experiment, carried out at 37 °C, gave identical results (results not shown). When complete binding curves were done in the presence of PMA, no appreciable difference in the dissociation constant was observed.

DISCUSSION

The phorbol ester tumour promoters have been shown to be potent activators of protein kinase C, and since the 'receptor' for the phorbols co-purifies with protein kinase C (Aschendel *et al.*, 1983), it has been suggested that activation of this enzyme is the mechanism by which these compounds alter cellular metabolism. Paradoxically, although protein kinase C activation is thought to be a normal component involved in the phosphoinositide pathway of cellular activation by calcium-mobilizing hormones and neurotransmitters, interaction of PMA with cells has been reported to block both the agonist-stimulated breakdown and resynthesis of the phosphoinositides (Sahai *et al.*, 1982; Smith *et al.*, 1983; Labarca *et al.*, 1984). We report here that such is also the case for vasopressin-sensitive WRK-1 cells. After a sufficient incubation time (25 min) with phorbols, inhibition in the range of 80% is observed; however, at earlier times (2 min) there is no significant interference with the hormone-induced rise in inositol phosphates or in cytosolic Ca²⁺. Down-regulation of vasopressin receptors is not seen in the presence of PMA, suggesting that the event involved in inhibition occurs subsequent to binding of ligand to receptor, perhaps through inactivation of the receptor coupling mechanism.

Recently, a number of investigators have reported that PMA can antagonize the effects of Ca²⁺-mobilizing hormones, including fMet-Leu-Phe (Naccache *et al.*, 1985), α_1 -adrenergic agonists (Corvera & Garcia-Sainz, 1984; Lynch *et al.*, 1985; Cooper *et al.*, 1985) and thrombin, platelet-activating factor and vasopressin (platelets) (MacIntyre *et al.*, 1985). Effects of vasopressin on liver have been reported to be unaffected by phorbol esters, except at low concentrations of vasopressin (Cooper *et al.*, 1985; Lynch *et al.*, 1985). Phorbol esters have minimal effects on vasopressin-induced phosphorylase activity, Ca²⁺-mobilization or inositol phosphate generation. The results presented here for short-term (2 min) incubations with PMA are in agreement with these findings. At early times, vasopressin activity is unaffected by the addition of tumour promoters; however, if one examines later times (25 min) one sees significant inhibition by these phorbols. The results shown in Table 3 indicate that the phorbols take a longer time to effectively inhibit phosphoinositide breakdown than does vasopressin to stimulate it. Thus either pretreatment for 25 min or simultaneous incubation for 25 min results in inhibition of vasopressin activity. The fact that the phorbols can inhibit the effects of other agonists, such as fMet-Leu-Phe, α_1 -adrenergic compounds and vasopressin (platelets), at early times may indicate either that these substances require longer times in order to activate phosphoinositide metabolism or that the early activity of PMA is powerful enough to obliterate a weaker (than the hormone) effect on phosphoinositide breakdown.

Lastly, OAG (100 ng/ml) was unable to mimic the inhibitory effect of PMA. Cooper *et al.* (1985), on the other hand, found that OAG could completely inhibit phenylephrine-induced rises in cytosolic free Ca²⁺, although much larger concentrations of OAG were required (12–60 μ g/ml) compared with PMA (10–100 ng/ml). We have no explanation for this discrepancy. We have also found that OAG is incapable of stimulating phosphatidylcholine metabolism in

WRK-1 cells, whereas PMA is a potent stimulator (results not shown).

In summary, phorbol ester tumour promoters are potent inhibitors of vasopressin-induced changes in phosphoinositide metabolism if given sufficient time to act. The delay in PMA activity may reflect the time required for the compound to penetrate the cell and reach its site of activity. Since we see no difference in the number of vasopressin receptors in the presence of PMA, we suggest that a decrease in the functionality of the receptor, due possibly to increased phosphorylation, as previously described for the insulin receptor (Takayama *et al.*, 1984) may explain the inhibitory effects of PMA on vasopressin activity.

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