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Control of glycogen phosphorylase interconversion by phorbol esters, diacylglycerols, Ca^{2+} and hormones in isolated rat hepatocytes

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In isolated rat hepatocytes: (1) phosphorylase activation by the ionophore A23187 was enhanced in the presence of tumour-promoting phorbol esters and 1,2- (but not 1,3-) diacylglycerols (dioleoyl- and oleoylacetyl-glycerol), with a similar dose-dependency; (2) the activation of phosphorylase by phenylephrine (1 μM) (but not by vasopressin or glucagon) was inhibited both by tumour-promoting phorbol esters and diacylglycerols, but with a different dose-dependency: complete inhibition was achieved with concentrations of phorbol esters two orders of magnitude lower than those of diacylglycerol; (3) binding of the α_1-adrenergic antagonist [^{3}H]prazosin and its displacement by unlabelled prazosin was not significantly affected in the presence of the phorbol esters. The possible involvement of protein kinase C in the control of phosphorylase interconversion is discussed.

INTRODUCTION

In rodent liver, as in many other tissues, the signal transduction of those hormones and agonists which act independently of cyclic AMP seems to involve both Ca^{2+} and the activation of a Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) (see Berridge, 1984; Nishizuka, 1984). Although Ca^{2+} mobilization is triggered by inositol 1,4,5-trisphosphate (Burgess *et al.*, 1984; Joseph *et al.*, 1984), the other moiety, 1,2-diacylglycerol, generated by the agonist-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate, may act as a separate messenger to activate protein kinase C. This property of diacylglycerol is mimicked by tumour-promoting phorbol esters (Castagna *et al.*, 1982; Kikkawa *et al.*, 1984); these phorboid compounds can thus conveniently be used to study the role of protein kinase C in cell activation. However, phorbol esters have been reported either to be without effect on phosphorylase interconversion in hepatocytes (Roach & Goldman, 1983; Garrison *et al.*, 1984) or to activate the enzyme synergistically with A23187 (Fain *et al.*, 1984) and to promote glucose production in the perfused liver (Kimura *et al.*, 1984). On the other hand, Corvera & García-Sáinz (1984) reported that the α_1-adrenergic stimulation of glucose production by hepatocytes was blocked by phorbol esters.

In the present work, the potential contribution of protein kinase C to the control of phosphorylase interconversion was evaluated by comparing the effects of phorbol esters and of 1,2-diacylglycerol in combination with the calcium ionophore A23187 and the α_1-adrenergic agonist phenylephrine.

MATERIALS AND METHODS

Hepatocytes

Hepatocytes were isolated from 200–280 g male fed Wistar rats between 10:00 and 11:00 h as described by Le Cam & Freychet (1977) and preincubated for 20 min at 37 °C in a Krebs–Henseleit (1932) bicarbonate buffer containing 3% (w/v) dialysed bovine serum albumin (Armour Pharmaceutical) and 10 mM-glucose. The hepatocytes were then washed and further incubated (50 mg of packed cells/ml) in the same buffer for 30 min, at which time, if not otherwise mentioned, hormones and other agents were added. Cell competence was assessed both by Trypan Blue exclusion (> 87%) and by hormone responsiveness (K_a values for phosphorylase activation by phenylephrine and glucagon) consistent with those previously reported (Hems & Whitton, 1980).

For the binding experiments, the hepatocytes were prepared as described above, diluted to 5 mg of packed cells/ml with incubation buffer and incubated in the presence of 40 nCi of [^{3}H]prazosin/ml with or without unlabelled prazosin or PMA (1 μg/ml) for 10 min at 37 °C (this time corresponded to maximal binding of the tracer to the cells). The cells were then diluted with 4 vol. of cold (0 °C) Krebs–Henseleit buffer without albumin and glucose, and rapidly (5 s) filtered under vacuum through Whatman glass microfibre filters (GF/C, 25 mm diameter). The filters were subsequently washed with 5 ml of the same cold buffer (additional washes did not decrease the non-specific binding further), dried, and their radioactivity was counted. [^{3}H]Prazosin bound was taken as an estimate of α_1-adrenergic receptors.

For measurement of phosphorylase *a*, batches of cells were frozen in liquid N_2 and later thawed in 3 vol. of 100 mM-NaF/20 mM-EDTA/0.5% glycogen in 100 mM-glycylglycine buffer, pH 7.4 (Stalmans *et al.*, 1974). Phosphorylase *a* activity was determined in these homogenates in the presence of 0.5 mM-caffeine, as well as total phosphorylase at 30 °C, as described by Stalmans & Hers (1975). A unit of phosphorylase is the amount of enzyme that converts 1 μmol of substrate/min in the conditions of the assay.

Materials

Collagenase (type 1), [arginine]vasopressin (grade VI), L-phenylephrine, 1,2- and 1,3-diolein, phorbol esters and bovine serum albumin were supplied by Sigma Chemical

Abbreviations used: PMA, 4β-phorbol 12β-myristate 13-acetate; OAG, 1-oleoyl-2-acetylglycerol.

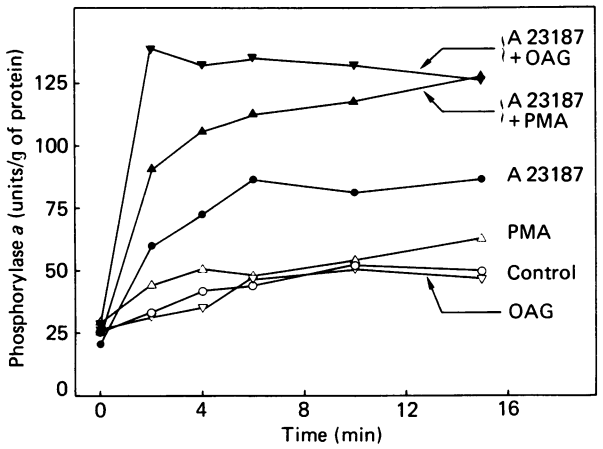

Fig. 1. Time course of phosphorylase activation by A23187 and synergism with PMA and OAG

Hepatocytes were prepared and incubated as described in the Materials and methods section. A23187 (2 μM), PMA (10 μg/ml) and OAG (25 μg/ml) were added at zero time alone or in combination as indicated. At various times after the additions, samples were taken and frozen pending phosphorylase *a* measurement.

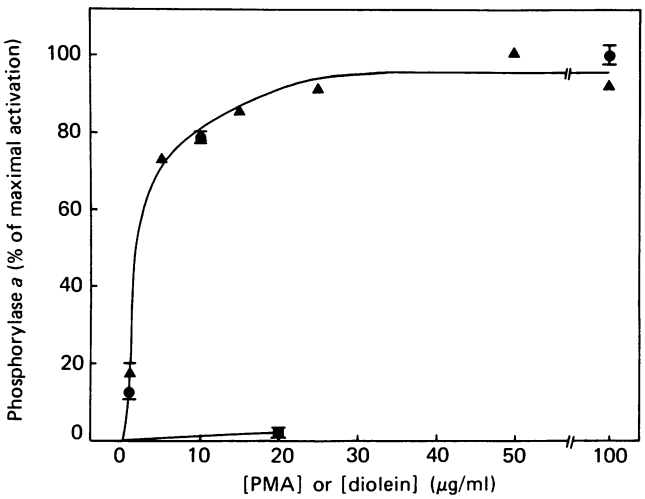

Fig. 2. Dose–response of phosphorylase activation by A23187 and PMA, 1,2- or 1,3-diolein

Hepatocytes were prepared and incubated as described in the Materials and methods section. A23187 was added at zero time alone or with various concentrations of PMA (●) and 1,2-diolein (▲) or with 20 μg of 1,3-diolein (■)/ml. Samples were taken 5 min later for phosphorylase *a* determination. Phosphorylase *a* activity is expressed as a percentage of maximal stimulation in the presence of 2 μM-A23187 and PMA or 1,2-diolein as indicated; 100% was equal to 87.5±2 (3) and 98 (2) units of phosphorylase *a*/g of protein (mean±S.E.M. for three and two different cell preparations in the presence respectively of 100 μg of PMA or 1,2-diolein/ml). Values are means for two cell preparations, or means±S.E.M. for three when indicated by bars.

Co. (St. Louis, MO, U.S.A.). Phosphorylase *a*, purified from skeletal muscle, was obtained from Boehringer (Mannheim, Germany). Pig glucagon was obtained from Novo Laboratories (Bagsvaerd, Denmark), and sodium pentobarbital (Nembutal) from Abbot Laboratories (Zug, Switzerland). The calcium ionophore A23187 was purchased from Calbiochem (La Jolla, CA, U.S.A.). OAG was generously given by Dr. H. D. Söling (Göttingen), and was further purchased from Molecular Probes Inc. (Junction City, OR, U.S.A.).

RESULTS

Synergistic activation of phosphorylase by A23187 and diacylglycerols or phorbol esters

The activation of phosphorylase by the Ca^{2+} ionophore A23187 was enhanced in the presence of PMA or OAG, whereas these compounds were inactive when added in the absence of the ionophore (Fig. 1). Similar results were obtained when OAG was replaced by 1,2-diolein (not shown). The extent of this synergistic activation of phosphorylase varied from one experiment to the other; for this reason the results in Fig. 2 were expressed as a percentage of maximal activation. The dose-dependency was the same for PMA or 1,2-diolein (Fig. 2); the 1,3-diolein isomer was, however, inactive, indicating a stereospecificity of the effect. A synergism between A23187 and PMA or 1,2-diolein on phosphorylase

Table 1. Specificity of the synergistic effect of A23187 and phorbol esters on phosphorylase activation in hepatocytes

Hepatocytes were prepared and incubated as described in the Materials and methods section. Saline or A23187, with or without phorbol esters, was added to the hepatocytes, and samples were taken 5 min later for phosphorylase *a* determination. Values are means±S.E.M. for two or three cell preparations. Phorbol esters added alone did not change the basal phosphorylase *a* values (see Fig. 1).

Addition	Phosphorylase *a* (units/g of protein)
NaCl (0.9%)	42±2 (3)
A23187 (2 μM)	83.5±2 (3)
A23187+active phorbol esters (10 μg/ml):	
PMA	102±7 (3)
4β-phorbol 12β, 13α-dibutyrate	102±5 (3)
4β-phorbol 12β,13α-dibenzoate	101 (2)
4β-phorbol 12,13-didecanoate	96.5 (2)
A23187+inactive phorbol ester (10 μg/ml):	
4β-phorbol 13α-monoacetate	82.5±5 (3)

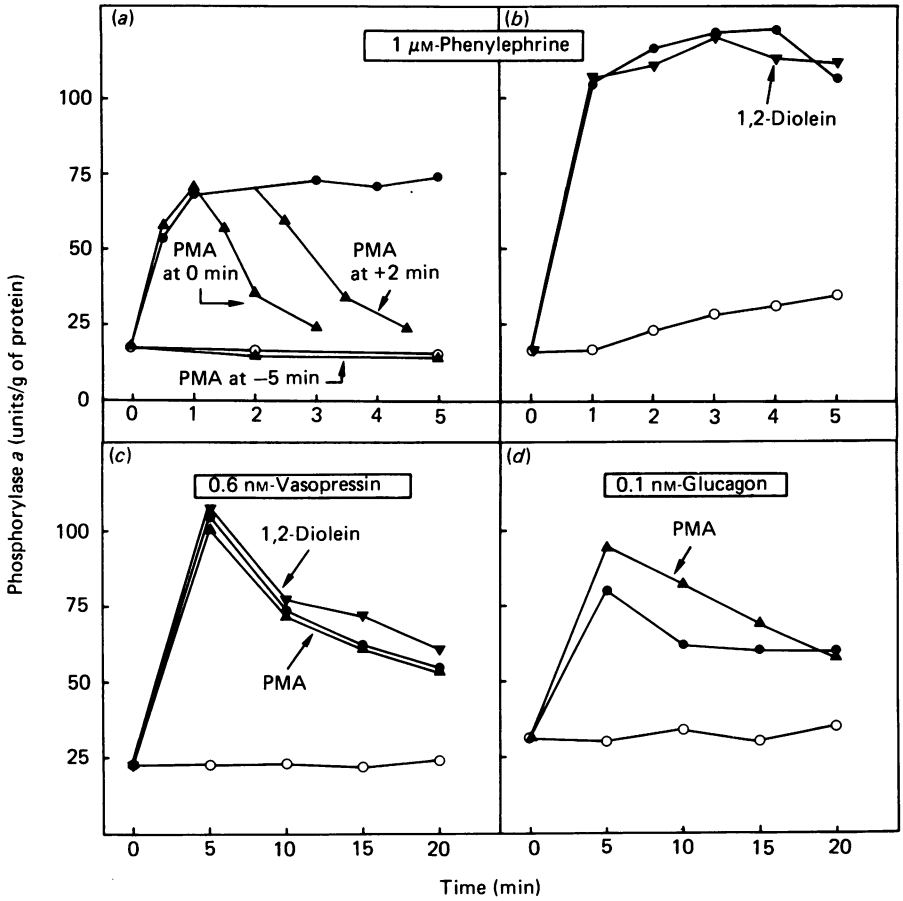

Fig. 3. Time course of phosphorylase activation by phenylephrine, vasopressin and glucagon: effect of PMA and 1,2-diolein

Hepatocytes were prepared and incubated as described in the Materials and methods section. Hormones alone (●) at the indicated concentrations, or saline (○), were added at zero time, and PMA (▲; 1 μg/ml) or 1,2-diolein (▼; 25 μg/ml) 5 min before the hormones, except where otherwise indicated.

activation was observed even in the presence of 10 μM of the α_1-adrenergic antagonist prazosin (results not shown), suggesting that it was not mediated by α_1-receptor binding and activation. The specificity of the phorbol esters' effect is illustrated in Table 1: only those esters having tumour-promoting activity were effective.

Antagonism of α_1-adrenergic activation of phosphorylase by OAG or PMA

The activation of phosphorylase by an optimal concentration (1 μM) of the α_1-adrenergic agonist phenylephrine was inhibited by PMA (1 μg/ml) (Fig. 3*a*): when the ester was added 5 min before the agonist, the inhibition was complete, when added together with phenylephrine, the response was short-lived, and when PMA was added 2 min after phenylephrine, phosphorylase was readily inactivated to control values. PMA had no effect on phosphorylase activation by vasopressin or glucagon when added 5 min before (Figs. 3*c* and 3*d*) or when added concomitantly with or after the hormones (results not shown). In contrast with the results obtained with PMA, a concentration of 1,2-diolein (25 μg/ml) that was optimal in provoking phosphorylase activation in combination with the ionophore (see Fig. 2) was unable to inhibit α_1-adrenergic activation of phosphorylase (Fig. 3*b*). Vasopressin activation of phosphorylase (Fig. 3*c*), as well as that of glucagon (results not shown), were unaffected by 1,2-diolein. Table 2 shows a similar specificity of tumour-promoting esters to inhibit phenylephrine activation of phosphorylase to that documented in Table 1 for the synergistic activation of phosphorylase by the esters and A23187. As illustrated in Fig. 4(*a*), the inhibition was dose-dependent for PMA ($K_i \simeq 10$ ng of PMA/ml). A 60% inhibition was also observed with OAG, but at a concentration (100 μg/ml) two orders of magnitude higher than that resulting in complete inhibition with the phorbol ester (Fig. 4*b*). The synergistic activation of phosphorylase by A23187 plus OAG (meant to mimic the effect of phenylephrine, but independently of α_1-receptor binding and activation) was, however, not inhibited by PMA (Fig. 4*a*), suggesting that the phorbol ester inhibited the binding of phenylephrine and/or interfered at an early post-receptor step leading to phosphorylase activation. The former possibility (inhibition by phorbol esters of phenylephrine binding) was next tested by using the α_1-adrenergic antagonist prazosin as a binding probe.

Effect of PMA on prazosin binding to hepatocytes

The binding of [^{3}H]prazosin and its displacement by increasing concentrations of non-radioactive prazosin were not significantly changed in the presence of the

Table 2. Effect of phenylephrine and specificity of phorbol esters' inhibition on phosphorylase activation in hepatocytes

Hepatocytes were prepared and incubated as described in the Materials and methods section. Phorbol esters or dimethyl sulphoxide (DMSO) were added to the cells 5 min before phenylephrine. Phosphorylase *a* was measured in samples of the hepatocytes taken 5 min after the addition of phenylephrine. Values are means ± S.E.M. for two or three cell preparations.

Addition	Phosphorylase *a* (units/g of protein)
NaCl (0.9%)	18 ± 3 (3)
DMSO (0.1%)	17.5 ± 1 (3)
Phenylephrine (1 μM)	56 ± 6 (3)
Phenylephrine + DMSO	55 ± 6.5 (3)
Phenylephrine + active phorbol esters (1 μg/ml):	
PMA	21 ± 1.5 (3)
4β-phorbol 12β,13α-dibutyrate	23.5 (2)
4β-phorbol 12β,13α-dibenzoate	27 ± 1 (3)
4β-phorbol 12,13-didecanoate	26 ± 1 (3)
Phenylephrine + inactive phorbol esters (1 μg/ml):	
4α-phorbol 12,13-didecanoate	54 ± 4 (3)
4β-phorbol 13α-monoacetate	46 ± 3.5 (3)

phorbol ester (Fig. 5). Also, PMA did not alter the binding of the tracer during an incubation at 37 °C for a period up to 60 min (results not shown). Non-specific binding (32.2 ± 1.4 and 35.7 ± 2% of maximal binding for control and PMA-treated hepatocytes respectively; mean values ± S.E.M. for four cell preparations) was estimated as the amount of tracer bound in the presence of 1 mM-prazosin. The binding parameters (time course of maximal binding and non-specific binding) reported in this work are in good agreement with earlier findings by Goodhardt *et al.* (1984).

DISCUSSION

Does protein kinase C activation lead to phosphorylase activation?

Studies by Garrison *et al.* (1984) suggest that the cyclic AMP-independent mechanism induced by hormones (vasopressin and angiotensin) in parenchymal liver cells involves the combined effect of the two messengers (inositol 1,4,5-trisphosphate and 1,2-diacylglycerol) generated by the hormone-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate and leading respectively to Ca^{2+} mobilization and protein kinase C activation. Our results show that both these signals might be important in the regulation of phosphorylase interconversion. The contribution of the former (increase in cytosolic Ca^{2+} concentration) to phosphorylase activation, which can be mimicked by the Ca^{2+} ionophore A23187, has already been well documented (Stubbs *et al.*, 1976; Pointer *et al.*, 1976; Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977) and is best explained by a stimulation of the Ca^{2+}-sensitive phosphorylase kinase (Shimazu & Amakawa, 1975; Khoo & Steinberg, 1975; van de Werve *et al.*, 1977; Vandenheede *et al.*, 1977; Sakai *et al.*, 1979; Doorneweerd *et al.*, 1982; Chrisman *et al.*, 1982). The contribution of the latter signal (protein kinase C activation) is strongly indicated by our findings and that of others (Fain *et al.*, 1984) that those phorbol esters active on protein kinase C, as well as the diacylglycerol activators (1,2-diolein and the analogue 1-oleoyl-2-acetylglycerol) of protein kinase C, potentiated phosphorylase activation by the Ca^{2+} ionophore. The effect of diolein was stereospecific, since 1,3-diolein was unable to enhance phosphorylase activation, in keeping with its lack of stimulation of purified protein kinase C (Rando & Young, 1984; Couturier *et al.*, 1984).

Kimura *et al.* (1984) observed a net increase in glucose production (presumably glycogenolysis) from livers perfused with Ca^{2+} and low concentrations of PMA (i.e. 10–100 ng/ml). In contrast, the concentrations of PMA needed in isolated hepatocytes to elicit a potentiation of phosphorylase activation by the Ca^{2+} ionophore A23187 were higher (above 1 μg/ml) (Fain *et al.*, 1984; the present work). This feature explains why other groups (Roach & Goldman, 1983; Garrison *et al.*, 1984), using lower concentrations of PMA (below 1 μg/ml), were unable to observe such a potentiation in hepatocytes. The reasons for this discrepancy between the results with intact liver and isolated hepatocytes is not clear.

The mechanism by which protein kinase C activation might lead to phosphorylase activation is unknown. The steady-state amount of phosphorylase in the active form is the result of the respective activities of phosphorylase kinase and phosphorylase phosphatase. An increased activation of phosphorylase can be the result of an increased phosphorylase kinase activity, a decreased activity of phosphorylase phosphatase, or both together. An activation of phosphorylase kinase by protein kinase C is unlikely, since phenylephrine does not induce such an activation (van de Werve *et al.*, 1977).

On the other hand, an allosteric inhibition of the phosphatase *in situ* remains a plausible hypothesis to explain phosphorylase activation by protein kinase C. Such an inhibition might be brought about by the appearance of a metabolite or protein, which, when phosphorylated by protein kinase C, could become inhibitory to phosphorylase phosphatase. The property of the deinhibitor protein of phosphorylase phosphatase (Defreyn *et al.*, 1977) to be inactivated by phosphorylation (Goris *et al.*, 1984) is interesting in this respect because its inactivation would lead to an inhibition of phosphorylase phosphatase. It remains, however, to be established if the deinhibitor protein can be inactivated by protein kinase C, as is the case with cyclic AMP-dependent protein kinase.

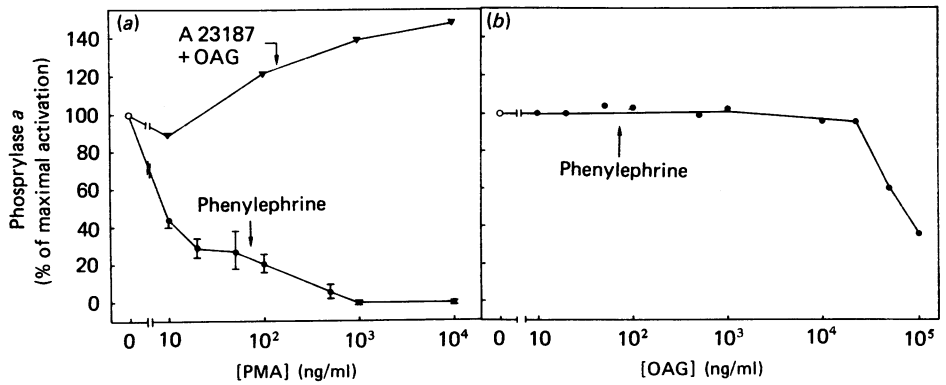

Fig. 4. Effect of various concentrations of PMA or OAG on the activation of phosphorylase by phenylephrine or OAG plus A23187

Hepatocytes were prepared and incubated as described in the Materials and methods section. (*a*) Various concentrations of PMA were added 5 min before the addition of phenylephrine (1 μM) or OAG (25 μg/ml) plus A23187 (1 μM) at zero time as indicated. (*b*) Various concentrations of OAG were added 5 min before the addition of phenylephrine (1 μM) at zero time. Samples were taken 5 min later for phosphorylase *a* determination. Phosphorylase *a* is expressed as the percentage of its maximal activation in the presence respectively of phenylephrine (absolute values were 68.3±3.8 units/g of protein; mean±S.E.M. for three cell preparations) or OAG plus A23187 (absolute values were 81 and 81.5 units/g of protein for two cell preparations). Values shown are means for two cell preparations, or means±S.E.M. for three, when indicated by bars.

Do phorbol esters and diacylglycerols inhibit α_1-adrenergic activation of phosphorylase through protein kinase C activation?

The present data show that the inhibition by phorbol esters of α_1-adrenergic stimulation of glucose production in isolated hepatocytes, previously reported by Corvera & García-Sáinz (1984), is due to an inhibition of phosphorylase activation. This inhibition is unlikely to be the result of altered binding of the agonist to the α_1-adrenergic receptor, since the phorbol PMA did not affect this parameter significantly (see Fig. 5 and the final paragraph in this section) at concentrations at which it completely blocked the activation of phosphorylase by phenylephrine (Fig. 4*a*). In contrast, PMA was unable to inhibit the activation of phosphorylase by the combined action of OAG and the Ca^{2+} ionophore (Fig. 4*a*). Taken together, these results indicate that the inhibition of phenylephrine activation of phosphorylase by PMA occurs at an early post-binding step preventing the expression of the message. In this hypothesis, the generation of both inositol 1,4,5-trisphosphate and diacylglycerol by phenylephrine should be impaired by PMA. An impairment of the Ca^{2+} branch (inositol 1,4,5-trisphosphate production) alone might, however, be sufficient to explain the inhibition by PMA of phenylephrine activation of phosphorylase (see also the final paragraph), because Ca^{2+} mobilization could be required for the expression of protein kinase C activity, as seems the case for phosphorylase activation. On the other hand, the fact that OAG or 1,2-diolein (which are presumed to activate protein kinase C similarly to PMA) had only an α_1-inhibitory effect at two orders of magnitude higher concentrations than the phorbol ester (Fig. 4*b*) is in keeping with the relative sensitivity of protein kinase C to these activators *in vitro* (Castagna *et al.*, 1982). Moreover, the specificity of the phorbol esters to inhibit α_1-activation of phosphorylase coincided with their reputed property to activate protein kinase C (Table 2).

In summary, the opposite effects on phosphorylase interconversion of phorbol esters (and diacylglycerol) might both be assumed to reflect protein kinase C activation. However, an increase in intracellular Ca^{2+} seems to be required for the potentiation of phosphorylase activation by protein kinase C, as evidenced by the lack of effect of phorbol esters and diacylglycerol in the absence of the Ca^{2+} ionophore A23187. In contrast, an

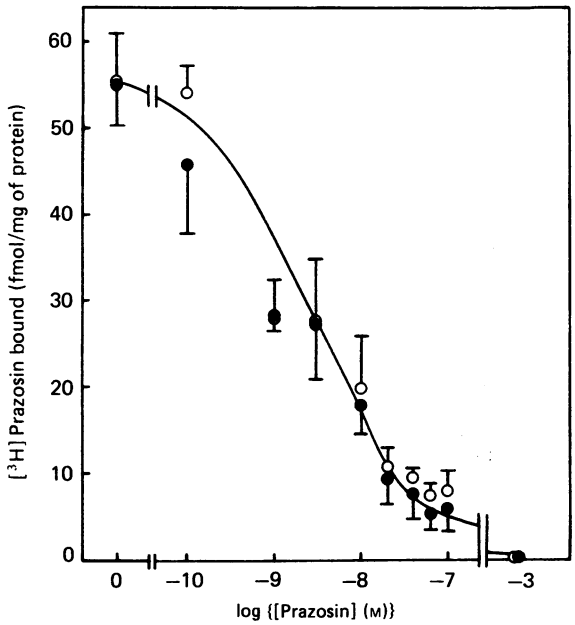

Fig. 5. Effect of PMA on prazosin binding to hepatocytes

Prazosin binding to hepatocytes was measured at 37 °C as described in the Materials and methods section. Hepatocytes were incubated with (●) or without (○) 1 μg of PMA/ml. Values are means±S.E.M. for four cell preparations. Statistical significance was assessed by Student's *t* test for comparison of paired and unpaired data: none of the binding values were different in the presence or absence of PMA at each prazosin concentration.

increase in Ca^{2+} is not required for the inhibitory effect of PMA or OAG on α_1-adrenergic action. This difference perhaps reflects different requirements for activation of protein kinase C depending on the cellular site that is affected, i.e. plasma membrane for the anti-α_1-adrenergic effect and cytosol for phosphorylase activation. Alternatively, it is interesting to recall, as we did previously (van de Werve *et al.*, 1985), that other receptors for phorbol esters than protein kinase C have been identified in A431 epidermic carcinoma membranes membranes (Mc n *et al.*, 1984). Such might also be the case for the liver α_1-adrenergic receptor.

While this work was being reviewed, observations consistent with ours were published, namely inhibition of phenylephrine activation, but not of glucagon activation, of phosphorylase by PMA without direct interference with [^{3}H]prazosin binding to purified plasma membranes, and dose-dependent inhibition of phenylephrine-stimulated increase in cytosolic free Ca^{2+} and inositol trisphosphate formation (Lynch *et al.*, 1985; Cooper *et al.*, 1985).

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