Regulation of hepatocyte plasma membrane α_1 -adrenergic receptors by 4 β -phorbol 12-myristate 13-acetate

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The effect of phorbol 12-myristate 13-acetate (PMA) on hepatocyte α_1 -adrenergic receptors was determined by [3 H]prazosin binding to plasma membranes from control and PMA-treated hepatocytes. Membranes from hepatocytes incubated with PMA (1 μ g/ml) for 1 h exhibited a 40% decrease in α_1 -adrenergic receptors (481 ± 10 fmol/mg of protein; mean $\pm$ S.E.M. for three separate experiments) relative to vehicle-treated (dimethyl-formamide) hepatocytes (802 ± 91 fmol/mg of protein; $n = 3$), with no significant effect on the K_D . The PMA-induced decrease in α_1 -adrenergic receptors was maximal by 30 min and half-maximal inhibition of [3 H]prazosin binding occurred with a PMA concentration of approx. 15 ng/ml. Pretreatment of hepatocytes with staurosporine (5 μ M) blocked the effect of PMA, and 4 β -phorbol 13-monoacetate was ineffective, suggesting the involvement of protein kinase C (PKC). Treatment of

hepatocytes with primaquine (300 μ M) for 15 min decreased hepatocyte plasma membrane α_1 -adrenergic receptors by 34.0 ± 2.4 % (mean $\pm$ S.E.M. of three experiments). Removal of primaquine allowed essentially complete recovery (98 ± 4 %; mean $\pm$ S.E.M. for five separate experiments) of plasma membrane [3 H]prazosin binding within 20 min, suggesting that the α_1 -adrenergic receptor undergoes endocytotic recycling. Addition of PMA (1 μ g/ml) to hepatocytes immediately after removal of primaquine, completely inhibited the increase in plasma membrane α_1 -adrenergic receptors relative to control cells, but had no effect on hepatocytes whose cell surface α_1 -receptors remaining after primaquine treatment had been inactivated by alkylation. These observations suggested that activation of PKC may facilitate the internalization of the α_1 -adrenergic receptor in hepatocytes.

INTRODUCTION

Phorbol esters have been shown to produce a rapid and profound inhibitory effect on the α_1 -adrenergic receptor-mediated increases in Ca^{2+} mobilization in hepatocytes (Cooper et al., 1985; Lynch et al., 1985a; van de Werve et al., 1985; Corvera et al., 1986), and smooth muscle cells (Leeb-Lundberg et al., 1985, 1987; Bouvier et al., 1987). Agonist-induced increases in cytosolic Ca^{2+} concentration occur in response to generation of $Ins(1,4,5)P_3$, following hydrolysis of membrane polyphosphoinositides by phospholipase C (Berridge, 1984; Williamson et al., 1985). A guanine nucleotide binding protein (G protein) couples activation of phospholipase C to agonist-receptor binding (Exton, 1993). Diacylglycerol, produced together with $Ins(1,4,5)P_3$, causes protein kinase C (PKC) activation (Nishizuka, 1986). Since phorbol esters mimic diacylglycerol in activating PKC (Castagna et al., 1982), and the cell-permeable diacylglycerol, 1-oleoyl-2-acetyl-glycerol, also inhibits the α_1 -adrenergic signalling pathway in hepatocytes (Cooper et al., 1985), it appears that PKC may play a role in receptor desensitization.

A number of studies have provided insight into the mechanism of the inhibitory effect of phorbol esters on the α_1 -adrenergic signalling pathway. Phosphorylation of the α_1 -adrenergic receptor has been demonstrated following addition of 4 β -phorbol 12-myristate 13-acetate (PMA) to ^{32}P -labelled DDT₁ MF-2 smooth muscle cells (Leeb-Lundberg et al., 1985), and by purified PKC *in vitro* (Bouvier et al., 1987). The existence of α_1 -adrenergic receptors in two different affinity states (Goodhart et al., 1982;

Lynch et al., 1985b; Corvera et al., 1986; Schwarz et al., 1986; Jagadeesh and Deth, 1987) appears to reflect whether or not they are coupled to the G protein involved in phospholipase C activation. A ternary (high affinity) complex consisting of agonist (hormone), receptor and G protein, is believed to be converted to a low-affinity (binary) state, following exchange of GTP for GDP and dissociation of the activated G protein, analogous to the role of G proteins in adenylyl cyclase activation (Casey and Gilman, 1988). Phorbol esters have been shown to convert α_1 -adrenergic receptors to the low affinity state in hepatocytes (Corvera et al., 1986; Beeler and Cooper, 1993), and bovine thoracic aorta (Jagadeesh and Deth, 1988). The rapidity of uncoupling of the α_1 -adrenergic receptor and G protein by phorbol 12,13-dibutyrate (PDBU), and the ability to reverse PDBU-induced uncoupling and to restore α_1 -adrenergic responsiveness, indicate that interaction between the α_1 -adrenergic receptor and G protein represents an important inhibitory locus for phorbol esters (Beeler and Cooper, 1993). However, the ability of phorbol esters to inhibit AlF_4^- -induced increases in $Ins(1,4,5)P_3$ and Ca^{2+} mobilization in hepatocytes (Blackmore and Exton, 1986), and to phosphorylate the phospholipase C- $\beta 1$ isoform in certain cells (Ryu et al., 1990), suggests an additional inhibitory locus for phorbol esters, distal to the α_1 -adrenergic receptor/G protein uncoupling mechanism.

Effects of phorbol esters on α_1 -adrenergic receptor binding capacity have also been reported. In plasma membranes prepared from liver perfused with PMA, the number of α_1 -adrenergic receptors, assayed by [3 H]prazosin binding, was reduced 30–44%

Abbreviations used: PMA, 4 β -phorbol 12-myristate 13-acetate; PKC, protein kinase C; G protein, guanine nucleotide binding protein; PDBU, 4 β -phorbol 12,13-dibutyrate; BCECF, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein.

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(Lynch et al., 1985a). Phorbol esters have also been shown to reduce α_1 -binding sites in smooth muscle (Cowlen and Toews, 1988; Jagadeesh and Deth, 1988), and cardiac muscle (Kushida et al., 1988). The present study was undertaken to further investigate the mechanism underlying the decrease in hepatocyte plasma membrane α_1 -adrenergic receptors, following exposure to PMA.

MATERIALS AND METHODS

Materials

Collagenase D was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.); fatty acid-free BSA (PENTEX, fraction V) was from Miles Diagnostics (Kankakee, IL, U.S.A.); A23187, 8-bromo-cyclic AMP, phorbol esters, primaquine, propranolol, Percoll and cycloheximide were from Sigma (St. Louis, MO, U.S.A.). Staurosporine was from Kamiya Biomedical (Thousand Oaks, CA, U.S.A.); leupeptin from Bachem (Torrance, CA, USA); phenoxybenzamine and prazosin were from Research Biochemicals (Natick, MA, U.S.A.); 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-dextran was from Molecular Probes (Eugene, OR, U.S.A.). [3 H]prazosin was purchased from DuPont/NEN (Boston, MA, U.S.A.). All other reagents were of analytical grade. Stock solutions of phorbol esters, A-23187, staurosporine, prazosin and phenoxybenzamine were prepared in dimethylformamide.

Preparation and incubation of hepatocytes

Isolated hepatocytes were prepared by collagenase perfusion of intact liver from fed, male, Sprague-Dawley rats (175–250 g), as described by Meijer et al. (1975). Hepatocytes were incubated (7.5 mg dry wt./ml) in Krebs-Ringer bicarbonate buffer containing 15 mM glucose and 0.2% (w/v) fatty acid-free BSA for the times indicated in the text or Figure legends at 37 °C in a water bath under an atmosphere of O₂/CO₂ (95:5).

Purification of plasma membranes from hepatocytes

Radioligand binding studies utilized plasma membranes prepared from hepatocytes incubated with phorbol esters or vehicle (dimethylformamide). The preparation of plasma membranes, based on Percoll density gradient centrifugation (Prpic et al., 1984) has been described previously (Beeler and Cooper, 1993). Protein content was determined by the procedure of Lowry et al. (1951) using BSA as standard. The specific activities of 5'-nucleotidase (Ipata, 1967) reflected a 19.6-fold purification of the membrane fraction (Beeler and Cooper, 1993), similar to results obtained for rat liver (Prpic et al., 1984).

Possible endosomal contamination of plasma membranes was assessed by incubating hepatocytes (10 mg dry wt./ml) in the presence or absence of BCECF-dextran (M_r = 40000; 1 mg/ml) for 15 min at 37 °C, to permit endocytosis. Hepatocytes were centrifuged (50 g, 90 s), resuspended in ice-cold buffer, and the washing process was repeated, prior to preparation of plasma membranes. Fluorescence measurements on the hepatocytes following homogenization and addition of Triton X-100 (0.1% v/v, final concn.) employing dual excitation at 439 nm and 505 nm, and measuring emission at 535 nm, showed a 2–3-fold increase in the fluorescence signal (excitation at 505 nm) from cells incubated with fluorescent dextran, relative to control cells. In three separate experiments, the maximum recovery of fluorescent probe in the plasma membrane fraction was approx. 3% relative to the homogenate; other values were not detectable and 1.5%. In contrast, recovery of 5'-nucleotidase activity in the

plasma membrane fraction was $32.7 \pm 3.1\%$ relative to the homogenate (mean $\pm$ S.E.M. from five separate experiments). Thus, there appeared to be relatively few endosomes associated with the purified plasma membrane fraction.

[3 H]prazosin binding to purified plasma membranes

Plasma membranes (15–35 μ g of protein/assay tube) were incubated for 30 min at 25 °C in a volume of 1.0 ml with varying concentrations of [3 H]prazosin (0.03–3 nM). The buffer contained 50 mM Tris/HCl (pH 7.4 at 25 °C), 10 mM MgCl₂, 1 mM EGTA, 3 mM catechol and 0.8 mM ascorbic acid; non-specific binding was determined in the presence of 10 μ M phentolamine. The assay was terminated by addition of 2 ml of ice-cold buffer (50 mM Tris/HCl, pH 7.4/10 mM MgCl₂) and filtration through glass fibre filter paper (Schleicher and Schuell, No. 34) using a Brandell Cell Harvester. Following washing of tubes and filters with 10 ml of ice-cold buffer, the filters were dried, transferred to vials containing Scintiverse BD, and radioactivity determined by liquid scintillation spectrometry. Determinations of [3 H]prazosin binding were made in quadruplicate or triplicate. Values for maximal binding (B_{max}) and K_D were calculated using a non-linear curve-fitting program (Lundon Software, Cleveland, OH, U.S.A.) based on the function:

$$B_s = \frac{B_{max} \times F}{K_D + F}$$

where B_s is the amount of specifically bound ligand, B_{max} is the maximal binding capacity, F is the free ligand concentration, and K_D is the apparent dissociation constant for [3 H]prazosin.

RESULTS

Effects of PMA on hepatocyte plasma membrane α_1 -adrenergic receptors

Binding of the α_1 -adrenergic receptor-selective radioligand, [3 H]prazosin, was used to quantify α_1 -adrenergic receptors in plasma membranes purified from hepatocytes by Percoll density gradient centrifugation (see the Materials and methods section). The specific binding of [3 H]prazosin to these membranes at 25 °C was rapid, with half-maximal binding occurring at 5 min and

Figure 1 Scatchard plot of [3 H]prazosin binding to plasma membranes from control and PMA-treated hepatocytes

Plasma membranes were prepared from cells incubated at 37 °C for 60 min with either 0.05% (v/v) dimethylformamide (○) or PMA (1 μ g/ml, ●). Plasma membranes (17 μ g and 22 μ g of membrane protein/assay tube for control and PMA-treated membranes, respectively) were incubated with [3 H]prazosin (0.03–3.0 nM) for 30 min at 25 °C and then filtered. Non-specific binding was determined in the presence of 10 μ M phentolamine. Data are from one representative experiment; each symbol represents the mean of triplicate determinations.

Figure 2 Time course of PMA effects on binding of [³H]prazosin to plasma membranes from PMA-treated hepatocytes

Hepatocytes were incubated at 37 °C with PMA (1 μ g/ml) for the indicated times prior to the purification of plasma membranes. For [³H]prazosin binding, plasma membranes from each time of PMA exposure (15–35 μ g of membrane protein/assay tube in three separate experiments) were incubated in triplicate with a saturating concentration of [³H]prazosin (1 nM) for 30 min at 25 °C. Values are expressed as % of control (100% corresponds to 721 $\pm$ 56 fmol/mg of protein). Data are means $\pm$ S.E.M. from three separate experiments.

maximal binding being attained by 15 min and remaining constant up to 45 min (results not shown). An incubation time of 30 min was utilized to achieve equilibrium binding. Specific [³H]prazosin binding was linear ($r = 0.998$) over the range 10–60 μ g of plasma membrane protein (results not shown).

Figure 1 shows a Scatchard plot analysis (Scatchard, 1949) of [³H]prazosin binding to plasma membranes prepared from hepatocytes exposed to vehicle (dimethylformamide, 0.05% v/v) or PMA (1 μ g/ml) for 60 min. Values ($\pm$ S.E.M.) for $B_{\max}$ (maximum number of binding sites) and K_D (apparent dissociation constant) for [³H]prazosin binding to control membranes obtained from three separate experiments, carried out as in Figure 1, were 802 $\pm$ 91 fmol/mg and 0.121 $\pm$ 0.024 nM, respectively. In membranes prepared from hepatocytes pretreated for 1 h with PMA (1 μ g/ml), values for $B_{\max}$ and K_D from three separate experiments were 481 $\pm$ 10 fmol/mg and 0.095 $\pm$ 0.017 nM, respectively. Thus pretreatment of hepatocytes for 1 h with PMA (1 μ g/ml) caused an approximate 40% decrease in the number of [³H]prazosin binding sites in plasma membranes prepared from such cells, with no significant effect on K_D .

The time course for manifestation of the effect of PMA is shown in Figure 2. It is evident that there is a progressive decrease in plasma membrane α_1 -receptors (measured by [³H]prazosin binding) in cells treated with PMA up to 15 min; thereafter the decrease becomes progressively slower and the number of α_1 -receptors remains unchanged between 30 and 60 min following PMA exposure. The dose–response relationship for this effect of PMA is shown in Figure 3, in which hepatocytes were incubated at 37 °C for 30 min with the indicated concentrations of PMA prior to plasma membrane purification and radioligand binding measurements. The concentration of PMA producing half-maximal inhibition of [³H]prazosin binding to plasma membranes was approx. 15 ng/ml and a maximal effect was attained with approx. 100 ng/ml.

Evidence for involvement of PKC in down-regulating α_1 -adrenergic receptors

Treatment of hepatocytes for 30 min with 4 β -phorbol 13-monoacetate (1 μ g/ml), a phorbol ester which does not activate

Figure 3 Dose–response relationship between PMA treatment and [³H]prazosin binding to plasma membranes purified from PMA-treated hepatocytes

Hepatocytes were preincubated at 37 °C for 30 min with the PMA concentrations indicated, prior to membrane purification. For radioligand binding, plasma membranes (15–30 μ g of membrane protein/assay tube in three separate experiments) were incubated in triplicate with 1 nM [³H]prazosin for 30 min at 25 °C. Values are expressed as % of control (100% corresponds to 647 $\pm$ 34 fmol/mg of protein). Data are means $\pm$ S.E.M. from three separate experiments. At the highest PMA concentrations (500 and 1000 ng/ml) values for [³H]prazosin binding (% of control) were 60.2 $\pm$ 1.4 and 61.9 $\pm$ 1.0, respectively; these error bars are too small to indicate on the Figure.

Figure 4 Inhibition of PMA-mediated decreases in [³H]prazosin binding by staurosporine

Cells were pretreated for 3 min with the indicated concentrations of staurosporine prior to the addition of PMA (1 μ g/ml) for 15 min. Membranes were purified and incubated with 1 nM [³H]prazosin for 30 min at 25 °C. [³H]prazosin binding to membranes prepared from vehicle and PMA-treated cells was 631 $\pm$ 21 and 492 $\pm$ 17 fmol/mg of protein, respectively. The 22.1% decrease in [³H]prazosin binding in the PMA-treated group represents 100% maximal PMA effects. Values are means of three separate experiments.

PKC (Castagna et al., 1982), produced no change in [³H]prazosin binding to plasma membranes relative to control (dimethylformamide)-treated cells, suggesting that activation of PKC is necessary for decreased binding to occur.

The effects of staurosporine, a PKC inhibitor (Tamaoki et al., 1986), on PMA-mediated decreases in [³H]prazosin binding sites were also examined (Figure 4). A 3 min pretreatment of cells with staurosporine prior to the addition of PMA (1 μ g/ml) inhibited the ability of PMA to decrease the number of α_1 -adrenergic receptors in hepatocyte plasma membranes in a dose-dependent manner, with half-maximal inhibition occurring at approx. 1 μ M.

The specificity of the decrease in [³H]prazosin binding to plasma membranes was further examined by treating hepatocytes with either 8-bromo-cyclic AMP or the Ca²⁺-ionophore, A23187,

Table 1 [^3H]prazosin binding to plasma membranes prepared from hepatocytes pretreated with either 8-bromo-cyclic AMP or A23187

Hepatocytes were incubated at 37 °C with either 100 μM 8-bromo-cyclic AMP or 1 μM A23187 for 5 or 15 min prior to the purification of membranes. For the radioligand binding assay, quadruplicate determinations were made for each treatment group by incubating membrane protein (15–35 μg /assay tube) with 1 nM [^3H]prazosin for 30 min at 25 °C. Data are means $\pm$ S.E.M. from three separate experiments.

Time of treatment (min)	Specifically bound [^3H]prazosin (fmol/mg)	
	8-bromo-cyclic AMP	A23187
0	742 $\pm$ 100	747 $\pm$ 35
5	714 $\pm$ 124	759 $\pm$ 58
15	701 $\pm$ 100	735 $\pm$ 47

Figure 5 Time course for the effects of phenylephrine and vasopressin treatment of hepatocytes on [^3H]prazosin binding to plasma membranes prepared from such cells

Hepatocytes were incubated at 37 °C with either 10 μM phenylephrine (Δ) or 100 nM vasopressin ($\circ$) for the indicated times prior to purification of the membranes. Plasma membrane protein (15–35 μg /assay tube in different experiments) was incubated with 1 nM [^3H]prazosin for 30 min at 25 °C in triplicate. Data are means $\pm$ S.E.M. from three experiments for vasopressin and four experiments for phenylephrine.

to effect activation of the intracellular cyclic AMP and Ca^{2+} messenger systems respectively. As shown in Table 1, neither agent produced any change in [^3H]prazosin binding to plasma membranes relative to control in hepatocytes pretreated with each agent for either 5 or 15 min. Control experiments involving measurement of phosphorylase *a* activities showed the expected increases in enzyme activity by either 8-bromo-cyclic AMP or A23187 (results not shown). It is therefore concluded that the ability to cause a decrease in [^3H]prazosin binding to plasma membranes is relatively specific for the PKC system and is not affected by intracellular Ca^{2+} or cyclic AMP levels.

On the basis of these observations, experiments were carried out to determine whether treatment of hepatocytes with either phenylephrine or vasopressin, which promote diacylglycerol formation secondary to phospholipid breakdown (Hughes et al., 1984; Bocckino et al., 1985; Williamson et al., 1985), could mimic the ability of PMA to decrease [^3H]prazosin binding to plasma membranes prepared from such cells. As shown in Figure 5, both phenylephrine (10 μM) and vasopressin (100 nM) produced a time-dependent inhibition of [^3H]prazosin plasma membrane binding. Maximal decreases for phenylephrine ($13.8 \pm 2.8\%$, mean $\pm$ S.E.M. for $n = 4$) and vasopressin

Table 2 Effect of agonist occupancy on PMA-induced decreases in [^3H]prazosin binding to plasma membranes prepared from treated hepatocytes

Hepatocytes were incubated at 37 °C for 5 min with 10 μM phenylephrine, for 5 min with 1 $\mu\text{g}/\text{ml}$ PMA, or for 5 min with 10 μM phenylephrine followed by a 5 min treatment with 1 $\mu\text{g}/\text{ml}$ PMA, prior to the purification of membranes. For radioligand binding measurements, plasma membranes (15–35 μg of membrane protein/assay tube in separate experiments) were incubated in triplicate with 1 nM [^3H]prazosin for 30 min at 25 °C. Data are means $\pm$ S.E.M. from four separate experiments.

Treatment	[^3H]prazosin bound (% of control)
Phenylephrine	87.9 $\pm$ 1.9
PMA	74.4 $\pm$ 1.0
Phenylephrine + PMA	74.5 $\pm$ 7.2

($23.3 \pm 1.2\%$, $n = 3$), which were statistically significant ($P < 0.05$), were produced after 5 min exposure, and thereafter there was a progressively smaller effect with time. These observations were consistent with a rather transient activation of PKC by diacylglycerol in response to the two agonists, in contrast with PMA, which produced a stable decrease in [^3H]prazosin binding for at least 60 min (see Figure 2), presumably reflecting a more sustained activation of PKC. The ability of phenylephrine itself to decrease [^3H]prazosin binding by direct competition in the subsequent radioligand binding assay appeared unlikely in view of the low affinity of phenylephrine for the α_1 -receptor and the number of steps involved in preparation of the membranes after centrifuging the hepatocytes to separate them from the phenylephrine-containing incubation medium.

In view of the above findings, a further experiment was carried out to determine whether agonist occupancy of the α_1 -receptor could be shown to affect the kinetics of the PMA-induced decrease in plasma membrane α_1 -receptors. [^3H]prazosin radioligand binding was carried out with membranes prepared from hepatocytes previously exposed to either phenylephrine (10 μM) for 5 min, PMA (1 $\mu\text{g}/\text{ml}$) for 5 min, or phenylephrine (10 μM) for 5 min followed by PMA (1 $\mu\text{g}/\text{ml}$) for a further 5 min. As shown in Table 2, phenylephrine pretreatment caused an approximate 12% decrease in [^3H]prazosin binding similar to that observed previously (Figure 5), while PMA pretreatment caused an approx. 25% decrease in [^3H]prazosin binding, comparable with that observed previously (Figure 2). However, when hepatocytes had been exposed to phenylephrine for 5 min followed by addition of PMA for a further 5 min there was no significant difference from the binding observed with PMA alone. These findings have supported the contention that the inhibitory effect due to phenylephrine itself is through a common mechanism with that induced by PMA and they appear to indicate that in the presence of an agonist, the effectiveness of PMA is unchanged.

Effects of phorbol esters on recycling of the α_1 -adrenergic receptor

Some insight into the basis for the decrease in the number of α_1 -receptors associated with the plasma membrane fraction from hepatocytes exposed to PMA, or to agonists which can elevate intracellular diacylglycerol, has come from experiments directed towards assessing receptor recycling in these cells. A number of cell surface receptors including those for low-density lipoprotein and asialoglycoprotein are known to undergo this process,

Figure 6 Effects of primaquine on [3 H]prazosin binding to membranes prepared from treated hepatocytes

(a) Time course for primaquine effects. Hepatocytes were incubated at 37 °C with primaquine (300 μ M) for the indicated times prior to purification of the membranes. For radioligand binding measurements, purified plasma membranes from each time of primaquine exposure were incubated in triplicate with 1 nM [3 H]prazosin for 30 min at 25 °C. Data are means $\pm$ S.E.M. from three separate experiments. (b) Effects of PMA on the reversibility of primaquine-induced decreases in [3 H]prazosin binding. Hepatocytes treated with primaquine (300 μ M) for 15 min at 37 °C were washed two times with Krebs–Ringer buffer at 4 °C and incubated at 37 °C in the presence (●) or absence (○) of PMA (1 μ g/ml). Plasma membranes were prepared from samples at the designated times and [3 H]prazosin binding to membranes was assayed. Values are expressed as % of control (100% corresponds to 720 ± 43 fmol/mg of protein). Data are means $\pm$ S.E.M. from five separate experiments.

involving endocytosis and the subsequent return of the receptor to the plasma membrane (Goldstein et al., 1985; Mellman, 1987). Weak bases such as primaquine and chloroquine inhibit receptor recycling by neutralizing the normally acidic endocytotic vesicles, thereby decreasing cell surface receptors (Schwartz et al., 1984; Mellman et al., 1986).

As shown in Figure 6(a), treatment of hepatocytes with primaquine (300 μ M) resulted in a rapid loss of plasma membrane α_1 -adrenergic receptors, with a maximal decrease (34%) occurring after approx. 10 min and remaining stable up to 30 min. The magnitude of the decrease (approx. 30%) was slightly less than that observed with a maximal dose of PMA (40–50% decrease; Figures 2 and 3), although the onset of the decrease was more rapid than with PMA (Figure 2). Treatment of hepatocytes for 15 min with PMA (1 μ g/ml) together with primaquine (300 μ M) decreased [3 H]prazosin binding to plasma membranes prepared from such cells, to a value of 51.0 ± 6.0 (% of control, mean $\pm$ S.E.M. for three separate experiments), compared with the value of 66.0 ± 2.4 (% of control; mean $\pm$ S.E.M. for three separate experiments) for primaquine (300 μ M) after 15 min. Thus, the effects of PMA and primaquine did not appear to be additive.

Evidence that the decrease in plasma membrane α_1 -adrenergic receptors observed in primaquine-treated hepatocytes reflects an effect on receptor recycling is shown in Figure 6(b), in which cells pretreated with primaquine for 15 min were subsequently washed and resuspended in fresh incubation medium. As shown by the open circles, there was almost full recovery of the original number of α_1 -receptor sites by 20 min after removal of the primaquine. Incubation of cells for 15 min with primaquine in the presence of the protein synthesis inhibitor cycloheximide (5 μ g/ml) produced a 36.7% decrease in [3 H]prazosin binding (mean of duplicate determinations). When cells were washed and incubated for 20 min in the continued presence of cycloheximide, 93.5% of the [3 H]prazosin binding sites were recovered (mean of duplicate determinations). These results suggested that the re-

Table 3 Reversibility of primaquine effects on [3 H]prazosin binding after inactivation of cell-surface α_1 -receptors

Cells were treated with primaquine (300 μ M) prior to the addition of prazosin (10 μ M) two times at 5 min intervals followed by phenoxybenzamine (50 nM) for 5 min. Cells were then diluted 1:1 with Krebs–Ringer bicarbonate buffer containing 5 mM dithiothreitol. The cells were washed twice and resuspended in fresh Krebs–Ringer bicarbonate buffer. Prior to incubation at 37 °C for 15 min, cells were treated with either PMA (1 μ g/ml) or vehicle (dimethyl-formamide). Data are means $\pm$ S.E.M. from three separate experiments. Statistical significance was determined by Student's *t* test: significantly different from control **P* < 0.05; different from prazosin/phenoxybenzamine treated group ***P* < 0.05.

Treatment	[3 H]prazosin bound (% of control)
Primaquine	$78.5 \pm 6.0^*$
Primaquine + prazosin + phenoxybenzamine	8.4 ± 4.9
Primaquine + prazosin + phenoxybenzamine/wash	$26.8 \pm 7.6^{**}$
Primaquine + prazosin + phenoxybenzamine/wash + PMA	$23.4 \pm 3.4^{**}$

covery of [3 H]prazosin sites detected after removal of primaquine was not due to *de novo* synthesis of α_1 -adrenergic receptors, but instead reflected a recycling of those α_1 -receptors whose return had been blocked by primaquine. In Figure 6(b), the inclusion of PMA (1 μ g/ml; solid circles) in the hepatocyte incubation medium, following washing to remove primaquine, totally inhibited the increase in plasma membrane α_1 -receptors relative to control levels. These observations suggested that PMA interfered with the normal pattern of α_1 -adrenergic receptor recycling, either to facilitate removal of the receptor from the membrane by endocytosis, or by inhibiting return to the cell surface.

To distinguish between these two possibilities, the experimental approach taken was to inactivate the remaining cell surface α_1 -adrenergic receptors following primaquine treatment, and after subsequently removing primaquine, the effect of PMA on membrane-associated α_1 -receptors was examined. The alkylating prazosin analogue, 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-bicyclo[2,2,2]octa-2,5-dienylcarbonyl)piperazine (prazosin), was used to irreversibly inactivate the α_1 -adrenergic receptor (Helman et al., 1987; Piascik et al., 1988). Treatment of hepatocytes with two successive additions of prazosin (10 μ M) for 5 min each, reduced [3 H]prazosin binding sites in purified plasma membranes by approx. 85%. A combination of prazosin (two successive additions of 10 μ M each for 5 min) followed by a low concentration of phenoxybenzamine (50 nM for 5 min), an irreversible antagonist of α -adrenergic receptors (Nickerson, 1949), was found to effectively decrease plasma membrane [3 H]prazosin binding sites by > 90%. As indicated in Table 3, treatment of hepatocytes with primaquine decreased [3 H]prazosin binding in plasma membranes by approx. 22% from control. Subsequent exposure of primaquine-treated hepatocytes to prazosin and phenoxybenzamine, under the conditions described previously, decreased plasma membrane [3 H]prazosin binding to 8.4 ± 4.9 % (mean $\pm$ S.E.M., for three separate experiments), relative to control. After extensive washing to remove primaquine and alkylating agents, hepatocytes were incubated in fresh medium in the presence or absence of PMA (1 μ g/ml) for 15 min.

Incubation in the absence of PMA resulted in an increase in plasma membrane [3 H]prazosin binding, reaching a value of 26.8 ± 7.6 % of control (mean $\pm$ S.E.M., for three separate experiments) compared with the value of 8.4 ± 4.9 % prior to washing and incubation. These results were consistent with a high return

(approx. 85%) of α_1 -adrenergic receptors to the plasma membrane following removal of primaquine. The extent of this increase was similar to that observed in Figure 6(b) in the absence of PMA, and in hepatocytes which had not been exposed to the alkylating agents, prazosin and phenoxybenzamine. Thus, it appeared that the conditions of exposure to the alkylating agents employed in Table 3, to irreversibly inactivate cell-surface α_1 -adrenergic receptors, did not have any appreciable effect on the apparent ability of α_1 -receptors, which had been internalized, to redistribute to the plasma membrane following removal of primaquine. Furthermore, from the data in Table 3, it appeared that there was no significant difference in binding of [3 H]prazosin to plasma membranes prepared from hepatocytes previously exposed to primaquine, prazosin and phenoxybenzamine and then washed and incubated for 15 min with either PMA or vehicle (dimethylformamide). Values for [3 H]prazosin binding to plasma membranes isolated from hepatocytes which had undergone these two treatment regimens involving washing and further incubation with PMA or dimethylformamide were $23.4 \pm 3.4\%$ and $26.8 \pm 7.6\%$ of control respectively (means $\pm$ S.E.M., for three experiments in each case). Since these results indicated that PMA did not affect reappearance of plasma membrane α_1 -adrenergic receptors following removal of primaquine from primaquine-treated hepatocytes, it appeared that the effect of PMA to decrease plasma membrane-associated α_1 -adrenergic receptors may have reflected an increase in the rate of endocytosis of the α_1 -receptor.

DISCUSSION

This study examined the effect of PMA on hepatocyte α_1 -adrenergic receptors by analysing the binding of the α_1 -selective radioligand, [3 H]prazosin, to plasma membranes prepared from control and PMA-treated hepatocytes. The characteristics of [3 H]prazosin binding to these plasma membranes from control hepatocytes ($B_{\max}$ and K_D values of 802 ± 91 fmol/mg, and 0.121 ± 0.024 nM, respectively; means $\pm$ S.E.M. for three separate experiments) were similar to those previously reported for [3 H]prazosin binding to plasma membranes prepared from rat liver, in which $B_{\max}$ values ranged from 975–1100 fmol/mg of protein, and K_D values were between 0.089 and 0.15 nM (Goodhart et al., 1982; Lynch et al., 1985a).

Treatment of hepatocytes with PMA for periods of 30 to 60 min resulted in a 40% decrease in [3 H]prazosin binding to plasma membranes from such cells, relative to control cells. The data of Figure 1 have indicated that this decrease in [3 H]prazosin binding reflects a reduction in the number of α_1 -adrenergic receptors, with no significant effect on K_D . The ability of PMA to activate PKC (Castagna et al., 1982) suggested that PKC may have been implicated in the mechanism underlying the decrease in hepatocyte plasma membrane α_1 -adrenergic receptors. This hypothesis was supported by the observations that pretreatment of hepatocytes with staurosporine (5 μ M), a PKC inhibitor (Tamaoki et al., 1986) blocked the ability of PMA to decrease plasma membrane α_1 -adrenergic receptors, and 4 β -phorbol 13-monoacetate, a phorbol ester which does not activate PKC (Castagna et al., 1982), was ineffective at decreasing plasma membrane α_1 -adrenergic receptors. Moreover, the ability to effect decreases in plasma membrane α_1 -adrenergic receptors appeared to be relatively specific to the PKC signalling pathway, since treatment of hepatocytes with either 8-bromo-cyclic AMP or with the Ca^{2+} -ionophore, A-23187, to activate the intracellular cyclic AMP and Ca^{2+} messenger systems, respectively, had no effect on [3 H]prazosin binding to plasma membranes prepared from such cells.

Treatment of hepatocytes with either phenylephrine or vasopressin produced a transient decrease in hepatocyte plasma membrane α_1 -adrenergic receptors, the maximal decrease occurring after 5 min with a return towards control levels thereafter (Figure 5). Such effects appeared to be consistent with the ability of phenylephrine and vasopressin to elevate intracellular diacylglycerol levels (Berridge, 1984; Hughes et al., 1984; Bocckino et al., 1985; Williamson et al., 1985), which in turn may have activated PKC. The more pronounced decrease in plasma membrane α_1 -adrenergic receptors with vasopressin may have reflected the increased diacylglycerol production by a maximally effective dose of vasopressin (Bocckino et al., 1985), which could result in greater activation of PKC. As regards the transient nature of the decrease in plasma membrane α_1 -adrenergic receptors by phenylephrine or vasopressin, Hughes et al. (1984) reported only a transient increase in hepatocyte diacylglycerol levels in response to vasopressin. Although Bocckino et al. (1985) observed a more sustained increase in hepatocyte diacylglycerol levels in response to vasopressin, the sustained second phase of hormone-stimulated diacylglycerol production in GH $_3$ cells did not activate PKC (Martin et al., 1990). Thus, the transient decreases in hepatocyte plasma membrane α_1 -adrenergic receptors elicited by phenylephrine and by vasopressin may have reflected a transient activation of PKC in contrast to the more sustained effects observed with PMA.

The decreases in [3 H]prazosin binding to plasma membranes following pretreatment of hepatocytes with phenylephrine or vasopressin are consistent with observations in DDT $_1$ MF-2 smooth muscle cells, in which agonists decreased cell surface α_1 -adrenergic receptors, as indicated by the binding of [3 H]prazosin to intact cells at 4 $^{\circ}\text{C}$ (Fratelli et al., 1987; Colucci et al., 1988; Cowlen and Toews, 1988). However, while the present study shows that PMA markedly decreases hepatocyte plasma membrane α_1 -adrenergic receptors, and also in livers perfused with PMA (Lynch et al., 1985a), it appears that in DDT $_1$ MF-2 cells PMA itself does not decrease plasma membrane α_1 -adrenergic receptors (Colucci et al., 1988; Cowlen and Toews, 1988), although PMA has potentiated the agonist-induced decrease in α_1 -adrenergic receptors (Cowlen and Toews, 1988).

Insight into the mechanism underlying the ability of PMA to decrease hepatocyte plasma membrane α_1 -adrenergic receptors in the present study has come from evidence indicating that the α_1 -adrenergic receptor undergoes constitutive recycling. It is well established that the receptors for transferrin and asialoglycoprotein cycle continuously, even in the absence of a bound ligand, by the process of endocytosis involving movement of receptors from coated pits on the cell surface into coated vesicles, and then into endosomes, from which receptors return to the cell surface (Goldstein et al., 1985). Treatment of hepatocytes with the weak base, primaquine, has elicited a rapid decrease in plasma membrane α_1 -adrenergic receptors, which is reversible upon washing the cells to remove primaquine (Figure 6). This effect is consistent with the ability of primaquine to cause a reduction in the number of asialoglycoprotein receptors on the cell surface of hepatoma cells (Schwartz et al., 1984), by neutralizing the normally acidic endocytotic vesicles and inhibiting receptor recycling (Mellman et al., 1986).

The effect of primaquine to decrease hepatocyte plasma membrane α_1 -adrenergic receptors was similar to that of PMA. The effects of PMA and primaquine were not additive, indicating that PMA may also exert an effect on the normal pattern of α_1 -adrenergic receptor recycling, although not necessarily at the same locus as primaquine. In hepatocytes exposed to primaquine and then washed, addition of PMA prevented the increase in plasma membrane α_1 -adrenergic receptors otherwise observed

upon removal of primaquine (Figure 6b). When the cell surface α_1 -adrenergic receptors remaining after primaquine treatment were inactivated by alkylation with prazosin and phenoxybenzamine, followed by extensive washing of the cells, addition of PMA had no effect on the increase in plasma membrane α_1 -adrenergic receptors following removal of primaquine. Since PMA did not appear to affect the return of α_1 -adrenergic receptors to the plasma membrane it may have acted either to facilitate removal of the receptor from the membrane, or in some manner analogous to primaquine, to inhibit the recycling process and cause accumulation of internalized receptors. As discussed previously, the effect of PMA appeared to be mediated by PKC activation, which in turn could promote phosphorylation of the α_1 -adrenergic receptor and/or some component of the endocytotic machinery and thereby have facilitated endocytosis. Alternatively, PKC activation may have prevented acidification of endosomes, albeit in a different manner from primaquine, and possibly involving an effect on the vacuolar H⁺-ATPase, responsible for acidification of endosomes (Forgac, 1989).

Previous studies from this laboratory (Beeler and Cooper, 1993) have shown that incubation of hepatocytes with PDBU promotes rapid uncoupling of the α_1 -adrenergic receptor and G protein (complete within 2 min), accompanied by inhibition of the α_1 -adrenergic signalling pathway, and both effects are reversible following removal of PDBU. Since the α_{1B} -adrenergic receptor sub-type present in rat hepatocytes and DDT₁ MF-2 smooth muscle cells (Minneman, 1988; Schwinn et al., 1991) has been shown to be phosphorylated by PMA in DDT₁ MF-2 cells (Leeb-Lundberg et al., 1985), it is likely that the phorbol-ester induced uncoupling of the α_1 -adrenergic receptor and G protein in hepatocytes involves PKC-induced phosphorylation of the receptor. The decrease in hepatocyte plasma membrane α_1 -adrenergic receptors by PMA in the present study occurs over a more extended time period (maximal decrease by 30 min) than the uncoupling of the α_1 -receptor and G protein. Moreover, it has previously been observed that pretreatment of hepatocytes with PMA (100 ng/ml) for 2 min completely inhibits the α_1 -adrenergic signalling pathway (Cooper et al., 1985), conditions under which a decrease of only approx. 20% of the hepatocyte plasma membrane α_1 -adrenergic receptors would be expected to occur. Thus it appears unlikely that the decreases in plasma membrane α_1 -adrenergic receptors observed in the present study play a significant role in the rapid desensitization of the α_1 -adrenergic signalling pathway by phorbol esters. Nevertheless, the marked decreases in cell surface α_1 -adrenergic receptors elicited by activation of PKC in response to phorbol esters or by agonist stimulation in this study indicate a role for PKC in regulating the distribution of the α_1 -adrenergic receptor. Further understanding of the mechanism underlying this decrease in cell surface α_1 -adrenergic receptors will be of interest in regard to the previously reported ability of PKC to promote rapid uncoupling of the α_1 -adrenergic receptor and G protein.

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