Mechanism of cellular effect of phorbol esters on action of arginine vasopressin and angiotensin II on rat vascular smooth muscle cells in culture

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The inhibitory effect of phorbol-12-myristate-13-acetate (PMA) on the Ca^{2+} -mobilization mechanisms by arginine vasopressin (AVP) and angiotensin II (AII) was analysed in rat vascular smooth muscle cells (VSMC) in culture. PMA inhibited the Ca^{2+} -mobilizing effect of both AVP and AII in a dose-dependent manner, including the rise in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) and Ca^{2+} efflux. In addition, inositol trisphosphate (IP_3) production induced by AVP or AII was more than 50% reduced by PMA. The involvement of protein kinase C was implicated by the diminution of the PMA effect by the specific protein kinase C inhibitor isoquinoline-sulphonyl-*O*-2-methylpiperazine (H7) and the lack of effect of an inactive phorbol. Thus, these results suggest that there is a blocking site that is common or similar for both AVP and AII signal transduction, and that it is a substrate for protein kinase C. This blocking action of protein kinase C occurred at least in part by inhibition of IP_3 production and, subsequently, a reduction in cytosolic Ca^{2+} release. In the presence of ionomycin, which produces an increase in $[\text{Ca}^{2+}]_i$ that is not altered by PMA, $^{45}\text{Ca}^{2+}$ efflux was increased instead of inhibited by PMA, thus suggesting that protein kinase C activation also stimulates a Ca^{2+} -extrusion mechanism in VSMC.

INTRODUCTION

The stimulation of protein kinase C by tumour-promoting phorbol esters has been demonstrated to interfere with the cellular action of several hormones, including angiotensin II (AII) (Brock *et al.*, 1985), norepinephrine and phenylephrine (Lynch *et al.*, 1985; Cooper *et al.*, 1985), arginine vasopressin (AVP) (Vittet *et al.*, 1986; Lassegue & Stoclet, 1986), bradykinin (Osugi *et al.*, 1987), acetylcholine (Vicentini *et al.*, 1986) and glucagon (Staddon & Hansford, 1986). The cellular mechanism(s) of this effect of phorbol esters is, however, unclear.

The effects of AVP, AII and several other hormones on vascular smooth muscle appear to be mediated by alterations in phosphoinositide metabolism and increases in cytosolic free Ca^{2+} , $[\text{Ca}^{2+}]_i$, and protein phosphorylation (Rasmussen, 1986; Abdel-Latif, 1986). The present studies have therefore examined the effect of phorbol esters on the AVP- and AII-induced changes in inositol trisphosphate (IP_3) levels, $[\text{Ca}^{2+}]_i$ and $^{45}\text{Ca}^{2+}$ efflux in rat vascular smooth muscle. Complementary studies with phorbol esters were performed in which $[\text{Ca}^{2+}]_i$ was increased by the Ca^{2+} ionophore, ionomycin, an agent that increases $[\text{Ca}^{2+}]_i$ mainly by a non- IP_3 -mediated mechanism (Kaibuchi *et al.*, 1983). Lastly, the role of protein kinase C versus other potential effects of phorbol esters on the action of AVP and AII was examined by administering phorbol esters in the presence and absence of the specific protein kinase C inhibitor, isoquinoline-sulphonyl-*O*-2-methylpiperazine (H7) (Hidaka *et al.*,

1984). Control studies were also undertaken with the inactive phorbol, 4- α -phorbol 12,13-didecanoate.

MATERIALS AND METHODS

Phorbol-12-myristate-13-acetate (PMA), 4- α -phorbol 12,13-didecanoate, H7, AVP and AII were purchased from Sigma and ionomycin from Calbiochem. [*myo*- ^3H]-inositol and labelled inositol phosphatides were obtained from Amersham.

Cell culture

Vascular smooth muscle cells (VSMC) were obtained from rat thoracic aortas as described by Chamley *et al.* (1977), by 2.5–3 h incubation in Eagle's minimum essential medium (MEM) containing 2 mg/ml collagenase (Cooper Biomedical). After washing to remove the collagenase, the freshly isolated cells were resuspended in Eagle's MEM containing 1 μM -glutamine, 100 U of penicillin G/ml, 100 μg of streptomycin/ml, and 10% (v/v) fetal bovine serum (FBS). Cells were plated in 9 cm² culture dishes at a density of 2×10^5 cells/1.5 ml of culture medium. For the Fura-2 experiments, cells were grown on round glass cover slips, 13 mm in diameter.

Cultures were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2 . Experiments were performed between 14 and 21 days of culture, when cells had formed a confluent monolayer with a density of approx. $(1.5\text{--}2) \times 10^6$ cells per dish. Identity of cells was

Abbreviations used: PMA, phorbol-12-myristate-13-acetate; AVP, arginine vasopressin; AII, angiotensin II; VSMC, vascular smooth muscle cells; MEM, minimum essential medium; H7, isoquinoline-sulphonyl-*O*-2-methylpiperazine; DMSO, dimethyl sulphoxide; FBS, fetal bovine serum; PSS, physiological saline solution; PBS, phosphate buffer solution; IP_1 , inositol monophosphate, IP_2 , inositol bisphosphate, IP_3 , inositol trisphosphate.

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confirmed by electron microscopy and viability was higher than 95%, as judged by Trypan Blue exclusion.

Measurement of $[Ca^{2+}]_i$

The monolayers on cover slips were incubated (for 45 min at 37 °C) with MEM containing no FBS and with 4 μ M-Fura-2 acetoxymethyl ester (AM). The monolayers were then washed three times with MEM containing no Fura-2 and incubated for a further 15 min at 37 °C to allow time for any non-hydrolysed Fura-2 AM to diffuse from the cells. Before measurement, the cover slips were rinsed again with physiological saline solution (PSS) containing 2 mM- Ca^{2+} . Fluorescence was measured at 37 °C using a fluorescence spectrophotometer equipped with a thermostatically-controlled cuvette holder (Perkin-Elmer 650-10S) at an emission wavelength of 500 nm and excitation wavelengths of 342 and 380 nm. Autofluorescence was measured in similar cells which had not been loaded with Fura-2 and was below 10% of the total fluorescence of Fura-2-loaded cells in all the experiments. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz *et al.* (1985).

Determination of $^{45}Ca^{2+}$ efflux

The culture medium was aspirated and cell monolayers were rinsed twice with 1 ml of PSS and loaded with 8 μ Ci of $^{45}Ca^{2+}$ in 1 ml of PSS at 37 °C for 3 h. The cultures were then rapidly rinsed 10 times (60 s total) with 1 ml of PSS. The medium was removed and replaced with 1 ml of PSS at 0, 1, 2, 3 and 4 min and thereafter at 30 s intervals. Each interval sample was placed into vials for liquid-scintillation counting (Packard Tri-Carb 460-C). Hormones were added at 6 min during the time course of $^{45}Ca^{2+}$ efflux. Intracellular radioactivity was extracted with 1 ml of SDS alkaline solution (0.1% SDS/2% (w/v) Na_2CO_3 /0.1 M-NaOH). The protein concentration was measured by the Lowry method (Lowry *et al.*, 1951). Results of $^{45}Ca^{2+}$ efflux were calculated as the percentage of the total cell's $^{45}Ca^{2+}$ counts released in 30 s. These counts were calculated as an average of the first four 30 s samples after the addition of the hormone.

In both Fura-2 and $^{45}Ca^{2+}$ efflux experiments, AVP, AII and ionomycin were used for cell stimulation; the doses are shown in the Results section. Cells were preincubated for 5 min before the addition of the hormone with PMA or vehicle [dimethyl sulphoxide (DMSO), final concentration < 0.01%], with and without the protein kinase C inhibitor, H7. Control experiments were also performed by preincubation of the cells with the inactive phorbol, 4- α -phorbol-12-13-didecanoate.

Inositol phosphate determination

After cells were grown to confluence, the culture medium in each dish was replaced with 1 ml of inositol-free medium containing 10 μ Ci of [*myo*-2- 3H]inositol (specific activity 16.3 Ci/mmol), and cells were incubated for 24 h. It has been previously shown that steady-state labelling occurs by 24 h (Emilsson & Sundler, 1984). Culture medium was aspirated and replaced with 500 μ l/dish of 37 °C phosphate buffer solution (PBS) containing the effectors. The reaction was stopped using 500 μ l of ice-cold trichloroacetic acid [20% (w/v) solution]. Cells were scraped off using an Eppendorf pipette top and aliquots were taken for protein determination.

The samples were centrifuged at 1000 *g* for 10 min.

The supernatant, containing inositol phosphates, was washed three times with an equal volume of ether and stored at -20 °C until analysis, which was performed during the same week as the extraction. The water-soluble fraction was thawed and brought to pH 6.0 using Tris (50 μ M). The extracts were applied to columns containing 1 ml of Dowex-1 ($\times 8$, formate form) and serially eluted with 1 ml aliquots of water, borax (5 mM-disodium tetraborate, 60 mM-sodium formate), 0.2, 0.5 and 1.0 M-ammonium formate (in 0.1 M-formic acid) separating, respectively, inositol, glycerophosphatidylinositol, inositol 1-phosphate, inositol bisphosphate, and inositol trisphosphates (Rhodes *et al.*, 1983); this last fraction includes 1,3,4- and 1,4,5-trisphosphates and 1,3,4,5-tetraphosphate. Samples were collected and counted in a scintillation counter. The extraction procedure has been previously validated with radioactive phosphoinositide. Results were expressed as number of IP_3 counts/ 10^4 total counts per mg of protein. For the experiments measuring inositol phosphates production, cells were stimulated with 10^{-7} M-AVP or 10^{-7} M-AII for 30 s and then the reaction was stopped. Experiments were performed both with and without 5 min preincubation with 10^{-7} M-PMA. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Statistical analysis

ANOVA and unpaired Student's *t* test were used for statistical comparisons.

RESULTS

$[Ca^{2+}]_i$ measurements

The results of Fura-2 experiments are shown in Table 1. Basal levels of $[Ca^{2+}]_i$ were 83.62 ± 3.05 nM ($n = 52$). Neither PMA nor its vehicle DMSO (final concentration < 0.01%) altered basal $[Ca^{2+}]_i$. DMSO also did not affect the AII- or AVP-mediated Ca^{2+} mobilization. PMA significantly inhibited the hormone-stimulated increase in $[Ca^{2+}]_i$ to a similar degree for both AVP and AII, as shown in Figs. 1*a* and *b*. The inactive PMA analogue 4- α -phorbol 12,13-didecanoate had no effect on either 10^{-7} M-AVP- ($n = 3$) or 10^{-7} M-AII- ($n = 3$)

Table 1. Suppression by PMA of AVP- and AII-induced increment in $[Ca^{2+}]_i$ (nM) and its partial reversal by H7

Increase in $[Ca^{2+}]_i$ levels (nM) from basal values by 10^{-7} M-AVP and 10^{-7} M-AII, both with and without 5 min preincubation with 10^{-7} M-PMA and with and without 5×10^{-5} M-H7 for 5 min preincubation followed by PMA for a further 5 min. Results are expressed as means $\pm$ S.E.M.

Preincubation conditions	Increase in $[Ca^{2+}]_i$ (nM)	
1 AVP ($n = 13$)	122.80 ± 9.05	} $P < 0.005$
2 PMA + AVP ($n = 10$)	29.78 ± 5.52	
3 H7 + PMA + AVP ($n = 4$)	$46.02 \pm 1.17^*$	} $P < 0.005$
4 AII ($n = 20$)	131.17 ± 14.99	
5 PMA + AII ($n = 11$)	28.97 ± 6.69	} $P < 0.0005$
6 H7 + PMA + AII ($n = 4$)	$57.62 \pm 7.62^\dagger$	

* $P < 0.25$, 3 vs. 1; $^\dagger P < 0.25$, 6 vs. 4.

Fig. 1. Inhibition by 10^{-7} M-PMA of the cytosolic Ca^{2+} increase produced by 10^{-7} M-AVP (a) and by 10^{-7} M-AII (b)

These typical experiments correspond to a minimum of 10 experiments showing similar results. (○), With PMA (10^{-7} M); (●), without PMA.

mediated $[Ca^{2+}]_i$ (results not shown). The maximal inhibitory effect of the response of the two hormones was observed after 5 min of preincubation with 10^{-7} M-PMA. No significant effect was detected at 1 min of preincubation with 10^{-7} M-PMA; 2 min of preincubation with 10^{-7} M-PMA produced 77.92% and 69.82% inhibition of the $[Ca^{2+}]_i$ increase induced by 10^{-7} M-AVP and AII, respectively. The protein kinase C inhibitor H7

Fig. 2. Effect of preincubation of cultured VSMC with different concentrations of PMA on the $^{45}Ca^{2+}$ efflux produced by 10^{-7} M-AVP and 10^{-7} M-AII

Each value corresponds to 3–10 experiments. * $P < 0.05$ compared with control without PMA. † $P < 0.025$ compared with control without PMA. ‡ $P < 0.005$ compared with control without PMA. (○), AII (10^{-7} M); (●), AVP (10^{-7} M).

partially reversed the effect of PMA (Table 1). H7 *per se* produced slight, brief changes in Fura-2 fluorescence, increasing basal $[Ca^{2+}]_i$ from 86 ± 12 to $108 \pm 8 \mu M$ ($P < 0.01$).

$^{45}Ca^{2+}$ -efflux

PMA inhibited AVP- and AII-stimulation of $^{45}Ca^{2+}$ efflux in a dose-dependent manner (Fig. 2). The time profile of this effect was similar to that described for $[Ca^{2+}]_i$ measurements, with a maximum PMA effect after 5 min of preincubation. H7, partially inhibited the PMA effect, as is shown in Table 2.

Ionomycin experiments

No interference by PMA in the amount of $[Ca^{2+}]_i$ released by ionomycin was observed, but the increase in $[Ca^{2+}]_i$ was less sustained in cells pretreated with PMA (Fig. 3). $^{45}Ca^{2+}$ efflux induced by 10^{-7} M-ionomycin was significantly increased by pretreatment with PMA (10^{-7} M) from 2.64 ± 0.32 to $4.35 \pm 0.4\%$ of total counts released/30 s ($P < 0.025$); $^{45}Ca^{2+}$ efflux induced by 10^{-8} M-ionomycin also increased after pretreatment with 10^{-7} M-PMA from 0.57 ± 0.035 to $2.12 \pm 0.12\%$ of total counts released/30 s ($P < 0.025$).

IP₃ measurements

As shown in Fig. 4, PMA (10^{-7} M) pretreatment significantly inhibited inositol phosphate production by AVP (10^{-7} M). AII- (10^{-7} M) induced inositol phosphate

Table 2. Effect of the protein kinase C inhibitor H7 on $^{45}Ca^{2+}$ -efflux inhibition by PMA

Concentration of AVP and PMA was 10^{-7} M and concentration of H7 was 5×10^{-5} M. Results are expressed as means $\pm$ S.E.M.

Preincubation conditions	$^{45}Ca^{2+}$ -efflux (% of total counts released/30 s)	
AVP ($n = 10$)	4.42 ± 0.52	} $P < 0.005$
AVP + PMA ($n = 10$)	1.17 ± 0.35	
AVP + PMA + H7 ($n = 4$)	2.89 ± 0.92	} $P < 0.025$

Fig. 3. Increase in $[Ca^{2+}]_i$ by ionomycin in cultured VSMC

Effect of 5 min pretreatment of the cells with 10^{-7} M-PMA ($n = 5$ experiments with PMA and $n = 5$ experiments without PMA). (●), Ionomycin (10^{-7} M); (○), PMA (10^{-7} M) + ionomycin (10^{-7} M). *, $P < 0.05$; **, $P < 0.01$.

Fig. 4. Effect of 5 min preincubation with 10^{-7} M-PMA on inositol phosphate levels after stimulation with 10^{-7} M-AVP ($n = 7$)

production (IP₁ 1015 ± 142 , IP₂ 443 ± 147 , IP₃ 253 ± 64.6 counts/10⁴ total counts per mg of protein, $n = 4$) was also decreased in the presence of PMA (IP₁ 634 ± 116.8 , IP₂ 266 ± 55.1 , IP₃ 117 ± 20 , all $P < 0.05$). When expressed as a percentage reduction, IP₁ was decreased by PMA pretreatment 48.0 and 37.5%, IP₂ 41.9 and 39.9% and IP₃ 58.9 and 53.7%, respectively for AVP- and AII-stimulated cells.

DISCUSSION

Although the cellular action of a number of hormones which are mediated by phospholipase C activation with subsequent production of IP₃ and diacylglycerol appears to be impaired by phorbol esters, the exact mechanism(s) of this blocking action remains unclear. In the present study, PMA markedly diminished the ability of both AVP and AII to increase $[Ca^{2+}]_i$ as measured by Fura-2. These results are very compatible with the previously reported observations that phorbol esters interfere with the AVP-induced cellular Ca^{2+} mobilization in hepatocytes (Cooper *et al.*, 1985), fibroblasts (Mendoza *et al.*, 1986) and vascular smooth muscle (Vittet *et al.*, 1986; Stassen *et al.*, 1987) as well as the effect of AII on vascular smooth muscle (Brock *et al.*, 1985).

The phorbol ester-mediated diminution in the rise in $[Ca^{2+}]_i$ induced by AVP or AII could have been due to an increase in cellular Ca^{2+} efflux, as shown in neutrophils by Lagast *et al.* (1984) and Rickard & Sheterline (1985), and in platelets by Pollock *et al.* (1987). The performance of $^{45}Ca^{2+}$ efflux studies, however, demonstrated that PMA reduced the increase in Ca^{2+} efflux normally observed during AVP and AII administration. This reduction in Ca^{2+} efflux was proportional to the observed reduction in intracellular Ca^{2+} peak. These results, however, do not necessarily conflict with those showing an increased Ca^{2+} efflux, since the decrease in Ca^{2+} efflux may have been secondary to diminished $[Ca^{2+}]_i$ release. In fact, our results using the Ca^{2+} ionophore, ionomycin, to release $[Ca^{2+}]_i$ support this interpretation. Specifically, the rise in $[Ca^{2+}]_i$ induced by ionomycin was shown to be associated with increased $^{45}Ca^{2+}$ efflux; pretreatment with PMA actually enhanced this ionomycin-induced Ca^{2+} efflux. Moreover, the levels of $[Ca^{2+}]_i$ during ionomycin induction, as measured by Fura-2, decrease more rapidly over time with PMA pretreatment. Taken

together, these results indicate interference by PMA in AVP- and AII-mediated $[Ca^{2+}]_i$ release, thereby accounting for the diminished cellular Ca^{2+} efflux. These events can obscure a direct enhancing effect of protein kinase C on Ca^{2+} efflux.

It seems reasonable to conclude that the effect of PMA is mediated as least in part by protein kinase C since the PMA effects on both AVP- and AII-induced cytosolic Ca^{2+} increase and Ca^{2+} efflux were partially reversed by H7, a specific inhibitor of protein kinase C, and were not mimicked by the inactive phorbol ester, 4- α -phorbol 12-13-didecanoate. It should be stated, however, that since the reversal of the PMA effects by H7 was not complete, a partial effect of H7 or protein kinase C-independent effects of PMA cannot be excluded, as suggested by Adunyah & Dean (1986).

Since PMA diminished the response to both AVP and AII in a similar manner, the present results are compatible with a structurally similar blocking site(s) which is presumably phosphorylated by protein kinase C. Since protein kinase C activation has been shown to interfere with IP₃ generation in different cellular, hormone-stimulated systems (Vittet *et al.*, 1986; Kojima *et al.*, 1986; Roth *et al.*, 1986; Pfeilschifter, 1986; Ting Xuan *et al.*, 1987; and the present study) the main blocking site(s) that is phosphorylated by protein kinase C is probably located in an activation step prior to IP₃ formation.

In this regard, phosphorylation of the α_1 -adrenergic receptor by protein kinase C and probably by different protein kinases has been proposed to be involved in homologous desensitization of these receptors (Leeb-Lundberg *et al.*, 1985; Lefkowitz & Caron, 1986). Brock *et al.* (1985) and Pfeilschifter (1986), however, did not find that phorbol esters altered AII-receptor binding kinetics. A second possible blocking site by protein kinase C-mediated phosphorylation may involve regulatory G proteins. Protein kinase C phosphorylates G proteins of the adenylate cyclase mechanism and modifies their activity (Katada *et al.*, 1985). In recent studies, G proteins were proposed to be the mediators in a protein kinase C-stimulated inhibition of AVP activation of phospholipase (Ting Xuan, 1987) and a role for a pertussis-sensitive G protein has been proposed for AII- and α_1 -adrenergic-signal transmission (Corda & Kohn, 1986; Bruns & Marme, 1987). Finally, a third possible site for a blocking action of protein kinase C is a phosphorylation-mediated, direct decrease in phospholipase C activity. As an additional mechanism for decreasing cellular response to hormones, protein kinase C-mediated phosphorylation of IP₃ phosphatase may occur, thereby accelerating IP₃ catabolism (Conolly & Majerus, 1986). Our results are consistent with this interpretation, showing a more marked decrease in IP₃ than in IP₁ and IP₂. As a final remark, although in the present study PMA produced a heterologous desensitization for both AVP and AII responses in the same cellular system, each hormone alone produced only homologous desensitization (C. Caramelo, K. Okada, P. Tsai & R. W. Schrier, unpublished results). Thus, there may be additional factors which differentiate the desensitization phenomena caused by hormone binding from that caused by phorbol esters.

This work was supported by grant DK19928 from NIH. C.C. was supported by a fellowship from the Ministry of Education and Science of Spain. The authors thank Dr.

Bradley S. Dixon for his valuable advice, Mrs. Carolyn Burke for illustrations and Ms. Linda M. Benson for secretarial assistance.

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Received 27 October 1987/15 March 1988; accepted 16 May 1988