Tumour promotor 12-*O*-tetradecanoylphorbol 13-acetate inhibits angiotensin II-induced inositol phosphate production and cytosolic Ca^{2+} rise in rat renal mesangial cells

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Preincubation of rat renal mesangial cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) strongly inhibited the increases of inositol phosphates and of free cytosolic Ca^{2+} induced by angiotensin II (10^{-7} M). TPA had no significant effect on the basal values of inositol phosphates and of free cytosolic Ca^{2+}. Inhibition appeared already after 1 min and was maximal after 5 min. These effects occur without significant changes on angiotensin II binding in intact cells. The concentration of TPA needed (10^{-9}–10^{-7} M) was in the range believed to cause specifically an activation of protein kinase C. Furthermore the biologically inactive phorbol ester 4α-phorbol 12,13-didecanoate was without effect. From the entirety of these results it is likely that protein kinase C inhibits angiotensin II activation of phospholipase C at a stage distal to receptor occupancy.

Angiotensin II *Inositol trisphosphate* *Ca^{2+}* *Phorbol ester* *Protein kinase C*

1. INTRODUCTION

A causal relationship between phosphoinositide turnover and Ca^{2+} mobilization induced by a number of hormones was first suggested by Michell in 1975 [1]. The mechanism, however, by which agonists mobilize Ca^{2+} was unclear for many years. Recently it has become evident that most agonists mobilize Ca^{2+} by stimulating the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane. A phospholipase C cleaves PIP_2 to yield inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DG) [2]. The IP_3 thus produced has been demonstrated to release Ca^{2+} from the endoplasmic reticulum and DG activates protein kinase C which in turn phosphorylates various cellular proteins [3,4]. There is good evidence that protein kinase C and cytosolic free Ca^{2+} may act independently or synergistically to initiate a wide variety of metabolic reactions [5]. Recent studies, however, have demonstrated that several agonist-induced processes in different cell types can be inhibited by preincubation with tumour-promoting phorbol esters, suggesting a role for protein kinase C as a negative feedback regulator of cell function [6–11].

Previously I have shown that vasoconstrictive hormones, like angiotensin II, arginine vasopressin or noradrenaline activate a phospholipase C in rat renal mesangial cells, thereby causing increased phosphoinositide hydrolysis and IP_3 and DG formation [12,13]. Both cleavage products play an important role in cellular responses in mesangial

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, *myo*-inositol trisphosphate; IP_2, *myo*-inositol bisphosphate; IP_1, *myo*-inositol monophosphate; DG, 1,2-diacylglycerol; quin 2/AM, 2-[(2-bis(carboxymethyl)amino-5-methylphenoxy)methyl]-6-methoxy-8-bis-(carboxymethyl)aminoquinoline tetrakis(acetoxymethyl) ester

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cells, especially in prostaglandin synthesis. Here, I used the mesangial cells to determine if phosphoinositide metabolism of Ca^{2+} mobilization are altered when 12-*O*-tetradecanoylphorbol 13-acetate (TPA) is used to activate protein kinase C. I report that pretreatment with TPA inhibits angiotensin II-stimulated IP_3 formation and Ca^{2+} mobilization in mesangial cells. These effects occur independently of significant changes in [^{125}I]angiotensin II binding capacity or affinity in intact cells.

2. EXPERIMENTAL

2.1. *Materials*

Angiotensin II, TPA and 4α-phorbol 12,13-didecanoate were from Sigma. 1-Oleoyl-2-acetylglycerol was from Molecular Probes. *myo*-[2-^{3}H]-Inositol was purchased from Amersham International. [*tyrosyl*-^{125}I]Angiotensin II was purchased from New England Nuclear. Quin 2/AM was from Calbiochem and all other chemicals from Merck.

2.2. *Cell culture*

Cultivation of rat mesangial cells was done as described [14]. For all experiments the first passage of mesangial cells was used. The cells were grown in RPMI 1640 (Boehringer) supplemented with 10% fetal bovine serum (Boehringer), penicillin (100 U/ml), streptomycin (100 μg/ml) (Boehringer) and bovine insulin at 0.66 U/ml (Sigma). Cells were incubated in a humidified atmosphere in incubators in air/CO_2 (19:1).

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

$[Ca^{2+}]_i$ was measured by using the fluorescent calcium indicator quin 2 as described [15]. In brief: about 2×10^7 cells were incubated with 25 μM quin 2/AM in RPMI 1640 for 20 min followed by another 40 min incubation with 4 vols of the medium. After the incubation period, aliquots of 1×10^6 cells were washed twice and resuspended in Hepes-buffered saline containing 1.3 mM Ca^{2+}. Fluorescence of quin 2-loaded cells was measured at 37°C in a Shimadzu RF-510 spectrofluorophotometer using an excitation wavelength of 340 nm and emission wavelength of 492 nm. The fluorescence signal was calibrated at the end of each individual trace essentially as described by Tsien et al. [16].

2.4. *Determination of inositol phosphates*

Mesangial cells were prelabelled for 2 × 72 h with *myo*-[2-^{3}H]inositol (10 μCi/ml) in MEM containing 10% dialysed fetal bovine serum. After the prelabelling period the medium was removed and the cells rinsed several times to remove free [^{3}H]-inositol. After this procedure cells were incubated for 5 min in 1 ml MEM with TPA or vehicle. Thereafter angiotensin II (10^{-7} M) was added. After 10 s the medium was aspirated and the reactions terminated by the addition of 1 ml of 15% (w/v) trichloroacetic acid. The different inositol phosphates were separated by anion-exchange chromatography on Dowex 1 X8 as in [13].

2.5. *Angiotensin II binding assay*

Binding experiments were done using dissociated cells scraped off the flasks as described [15]. In brief: [^{125}I]angiotensin II was added at 0.5 nM and incubated with 30–40 μg cellular protein per tube at room temperature for 40 min. At the end of the incubation, bound radioactivity was separated through a Millipore filter (0.22 μm GS). An aliquot of the cell suspension was used for protein determination [17]. Specific binding was calculated by subtracting the binding in the presence of 5 μM

Table 1

Effect of TPA and 1-oleoyl-2-acetylglycerol on angiotensin II-induced formation of inositol phosphates

Addition	^{3}H radioactivity (cpm/mg protein) in		
	IP_1	IP_2	IP_3
Control	1762 ± 115	557 ± 38	528 ± 43
Angiotensin II	3172 ± 242	1437 ± 95	1146 ± 52
TPA	1812 ± 95	538 ± 22	540 ± 37
Angiotensin II + TPA	2150 ± 212	796 ± 64	614 ± 34
OAG	1822 ± 152	573 ± 44	552 ± 63
Angiotensin II + OAG	2413 ± 203	895 ± 63	722 ± 43

Mesangial cells were prelabelled with *myo*-[^{3}H]inositol, treated with TPA (10^{-7} M, 5 min), 1-oleoyl-2-acetylglycerol (OAG) (5×10^{-6} M, 5 min) or vehicle and then exposed to angiotensin II (10^{-7} M) for 10 s. Data are means ± SE of 4 experiments

unlabelled angiotensin II from total binding and expressed as fmol bound angiotensin II/mg cell protein.

3. RESULTS

Stimulation of mesangial cells prelabelled with [^{3}H]inositol with angiotensin II (10^{-7} M) for 10 s caused a significant increase in formation of IP_1 (+80%), IP_2 (+158%) as well as IP_3 (+117%) (table 1). Preincubation of mesangial cells for 5 min with TPA (10^{-7} M) had no significant effect on the basal inositol phosphates but nearly totally inhibited angiotensin II-induced increases in IP_1, IP_2 and also IP_3 (table 1). This effect of TPA was dose-dependent (fig.1, upper) and also dependent on the preincubation time (fig.1, lower). The non-tumour-promoting 4α-phorbol 12,13-didecanoat had no effect on angiotensin II-stimulated IP_3 for mation as shown in fig.1. The synthetic DG analogue 1-oleoyl-2-acetylglycerol also significantly inhibited the angiotensin II-induced inositol phosphate production (table 1). Using quin 2-labelled mesangial cells, suspended in a medium of $[Ca^{2+}]_o$ = 1.3 mM, angiotensin II (10^{-7} M) caused an elevation of $[Ca^{2+}]_i$ from a resting value of 199 + 4 nM (mean ± SE, n = 17) to a maximum of 382 + 41 nM (mean ± SE, n = 9). Pretreatment with TPA (10^{-7} M, 5 min) did not alter the basal level of $[Ca^{2+}]_i$: 192 + 15 nM (mean ± SE, n = 5) but dose- and time-dependently inhibited the angiotensin II-stimulated increase in $[Ca^{2+}]_i$ (figs 2,3). The biologically inactive 4α-phorbol 12,13-didecanoate was without effect (fig.3).

To exclude the possibility that TPA-induced attenuation of the angiotensin II effects is due to decreased density or affinity of receptors on mesangial cells we determined the specific binding of [^{125}I]angiotensin. The specific binding of [^{125}I]angiotensin II to intact mesangial cells was

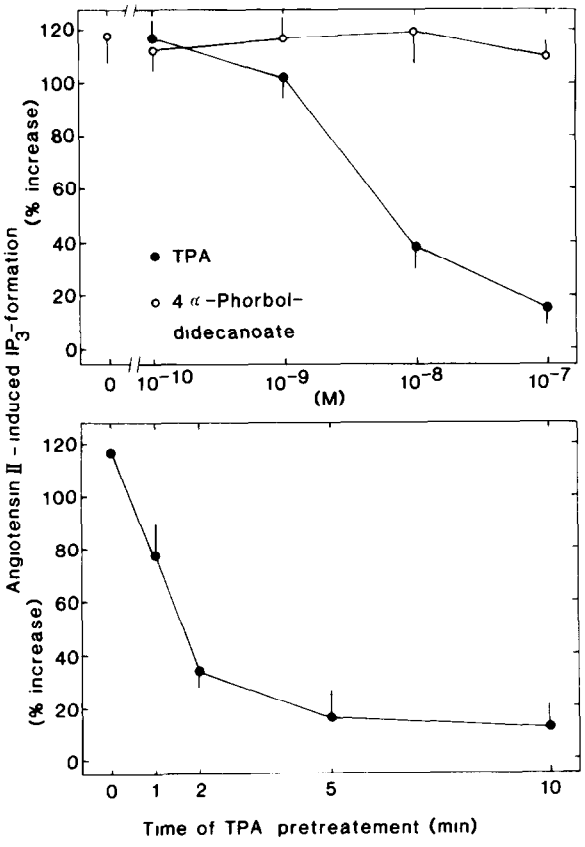

Fig.1. Time and dose dependency of inhibition of angiotensin II-induced IP_3 formation by TPA or 4α-phorbol 12,13-didecanoate. Values are means from 3–4 experiments ± SE.

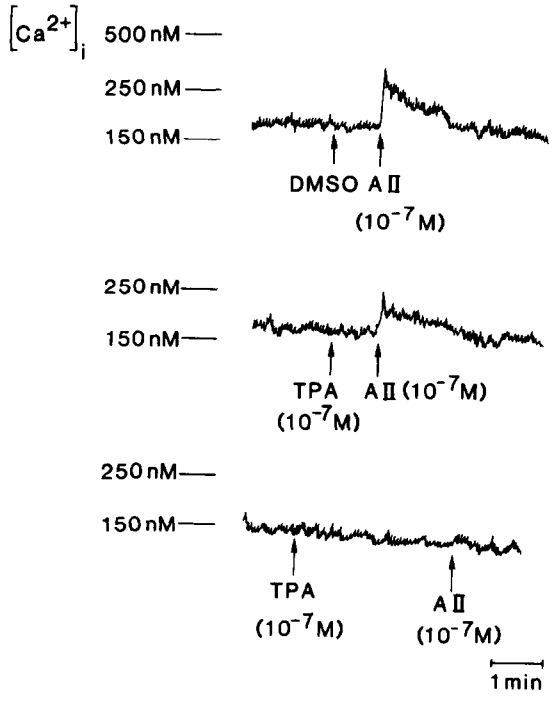

Fig.2. Effect of TPA on angiotensin II-induced increases in $[Ca^{2+}]_i$ as indicated by quin 2 fluorescence.

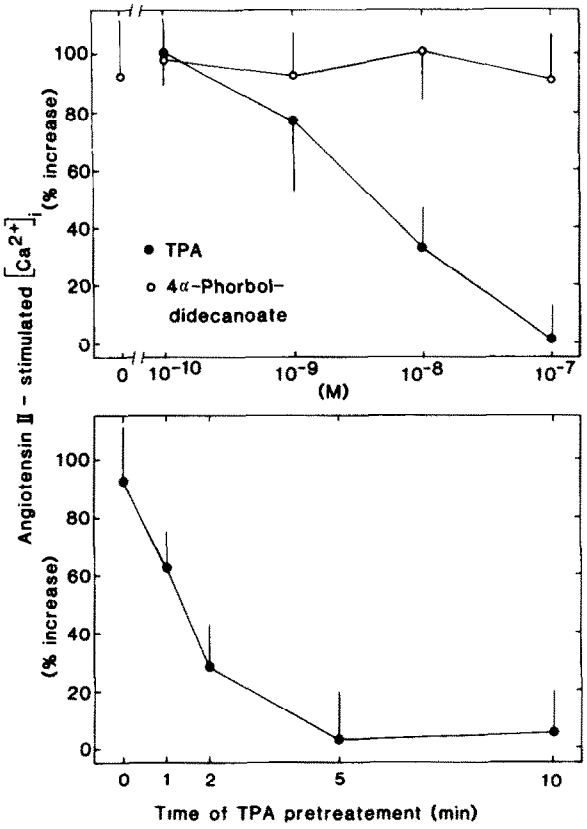

Fig.3. Time and dose dependency of inhibition of angiotensin II-induced increases in $[Ca^{2+}]_i$ by TPA or 4α-phorbol 12,13-didecanoate. Values are means from 3–17 experiments ± SE.

plotted as a function of the concentration of the labelled hormone. A linear curve was obtained corresponding to one group of receptor sites. The maximal binding sites (39 fmol/mg protein) and the apparent dissociation constant K_d (2.1 nM) were calculated. There was no significant difference in either the number of binding sites or their affinities for angiotensin II between control cells and cells treated with TPA (10^{-7} M) for 5 min.

4. DISCUSSION

The inhibition of angiotensin II-induced PIP_2 hydrolysis and $[Ca^{2+}]_i$ rise by TPA in mesangial cells occurred at the low concentrations of the phorbol ester which are thought to be specific for activation of protein kinase C. Also, the effect of 1-oleoyl-2-acetylglycerol on angiotensin II-stimulated IP_3 formation and the ineffectiveness of 4α-phorbol 12,13-didecanoate suggest that phosphorylations by protein kinase C might mediate these effects of TPA. Our results indicate that TPA did not modulate the binding properties of the angiotensin II receptor in cultured mesangial cells. This is in contrast to observations reporting that phorbol esters decrease either the binding capacity or the affinity for several hormones, including α_1-adrenergic ligands in hepatocytes [9], somatostatin binding in GH_3 pituitary cells [18] and insulin in cultured blood cells and fibroblasts [19,20]. On the other hand, the binding of vasopressin in hepatocytes [9], of fMet-Leu-Phe in neutrophils [8], of insulin in hepatoma cells [21] and of angiotensin II in vascular smooth muscle cells [11] is unaffected by TPA. In the latter cells as well as in mesangial cells the inhibition induced by TPA occurred at a stage distal to receptor occupancy. The mechanism underlying these inhibitory effects of TPA has not been defined directly.

Recently, I have shown that a G-protein is likely to be involved in coupling of angiotensin II receptor to phospholipase C activation in mesangial cells [15]. Pretreatment of mesangial cells with *Bordetella pertussis* toxin inhibits angiotensin II-stimulated effects in mesangial cells. There are striking similarities in the action of TPA and pertussis toxin in mesangial cells: both inhibit dose- and time-dependently phosphoinositide hydrolysis and $[Ca^{2+}]_i$ rises without affecting angiotensin II binding to intact mesangial cells. Therefore it is tempting to speculate that TPA and pertussis toxin may have the same target, i.e. a G-protein coupling angiotensin II receptors to phospholipase C. Pertussis toxin encouples the receptor by ADP-ribosylation of this G-protein [15] and TPA activates protein kinase C which perhaps phosphorylates and thereby also inactivates this G-protein. In addition, Katada et al. [22] have demonstrated that protein kinase C is able to phosphorylate the inhibitory guanine-nucleotide-binding regulatory component of adenylate cyclase, thereby suppressing its function. Garrison et al. [23] have reported that phorbol esters increase the phosphorylation of a 35 kDa protein which is also phosphorylated by angiotensin II. Interestingly, this protein is of similar size to the β-subunit of

G_s and G_i in the adenylate cyclase system [24]. Such a modulation of signal transduction in calcium-mobilizing cells by protein kinase C at the level of regulatory G-proteins would also explain the observed heterologous desensitization of angiotensin II by arginine vasopressin in mesangial cells (Pfeilschifter, J., unpublished).

I am currently trying to characterize further the possible target substrate of protein kinase C which enables the enzyme to play a dual role in cellular activation: not only to mediate but also to terminate cellular responsiveness initiated by exposure to agonists.

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