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Potentiation of angiotensin II-stimulated phosphoinositide hydrolysis, calcium mobilization and contraction of renal mesangial cells upon down-regulation of protein kinase C

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Long-term pretreatment of rat mesangial cells with 12-O-tetradecanoylphorbol 13-acetate (TPA) down-regulated protein kinase C activity and potentiated the angiotensin II-induced inositol trisphosphate ($InsP_3$) formation. This increased response to angiotensin II occurred without a significant change in the receptor number or K_d value of angiotensin II binding to the cells. The biologically inactive phorbol ester 4α-phorbol 12,13-didecanoate was without effect on angiotensin II-stimulated $InsP_3$ generation. Long-term pretreatment with TPA also increased the angiotensin II-induced mobilization of Ca^{2+} and the subsequent contraction of mesangial cells.

Angiotensin II; Phosphoinositide; Ca^{2+}; Protein kinase C; Contraction; Mesangial cell

1. INTRODUCTION

Mesangial cells are a major determinant in the regulation of the glomerular filtration rate [1]. Morphologically, mesangial cells resemble vascular smooth muscle cells and are able to contract upon stimulation by vasoactive hormones like angiotensin II [2–4]. However, the hormone fails to produce a sustained contraction of the cells, reflecting a specific, homologous desensitization phenomenon [3–5]. The direct action of angiotensin II on mesangial cells results in a rapid formation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DG), which are concomitantly generated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate [6–8]. $InsP_3$ mobilizes Ca^{2+} from endoplasmic reticulum, while DG activates protein kinase C [9,10]. These biochemical signals then interact to cause contraction of mesangial cells [7,8]. Angiotensin II-, vasopressin- and ATP-induced generation of $InsP_3$ and subsequent Ca^{2+} mobilization have been shown to be decreased or abolished by short-term preincubation with 12-O-tetradecanoylphorbol 13-acetate (TPA) [11–13]. Two further approaches can be chosen in order to elucidate the contribution of protein kinase C to the feedback inhibition of angiotensin II-induced phosphoinositide hydrolysis. One approach is the use of inhibitors of protein kinase C, which is limited, however, by the partial selectivity of drugs [5]. Alternatively, long-term exposure of mesangial cells to TPA results in a down-regulation of protein kinase C [6]. In this report we show that down-regulation of protein kinase C results in elevated basal and angiotensin II-stimulated levels of $InsP_3$, and subsequent amplified Ca^{2+} mobilization and contraction of mesangial cells.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Angiotensin II, TPA and 4α-phorbol 12,13-didecanoate were purchased from Sigma, St. Louis, MO; angiotensin II was also obtained from Bachem, Bubendorf, Switzerland; myo-[2-^{3}H]inositol was purchased from Amersham International, U.K.; [tyrosyl-^{125}I]angiotensin II from Anawa, Wangen, Switzerland; fluo-3/AM from Molecular Probes, Eugene, OR; ionomycin was from Calbiochem, Lucerne, Switzerland; all cell culture nutrients were from Boehringer Mannheim, FRG; all other chemicals used were from Merck, Darmstadt, FRG.

2.2. *Cell culture*

Rat mesangial cells were cultivated as described previously [14]. The cells were grown in RPMI 1640 supplemented with 20% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and bovine insulin at 0.66 unit/ml (Sigma). Mesangial cells were characterized morphologically by phase contrast microscopy, positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells [15], negative staining for factor VIII related antigen and cytokeratin, excluding endothelial and epithelial contamination, respectively.

2.3. *Cell labelling*

Confluent mesangial cells in 35 mm diameter dishes were labelled for 72 h with myo-[2-^{3}H]inositol (10 μCi/ml) in RPMI 1640 free of inositol, containing 2% dialyzed fetal calf serum. Phorbol esters or vehicle were added for the last 24 h of the labelling period.

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2.4. *Extraction and determination of inositol phosphates*

After the labelling period, the medium was removed and the cells were rinsed several times to remove free [^{3}H]inositol and incubated for an additional 1 h in fresh medium. After this procedure mesangial cells were incubated in 1 ml RPMI 1640 with or without angiotensin II for the indicated time periods. Thereafter the reaction was termined by rapid aspiration of the medium and addition of 1 ml of 15% (w/v) trichloroacetic acid. For extraction of inositol phosphates the dishes were put on ice for 1 h. The trichloroacetic acid was then removed with diethyl ether. The final extract was neutralized and applied to anion exchange columns containing 1 ml of Dowex 1-X8 (100–200 mesh, formate form; Serva, Heidelberg, FRG). Free inositol and the inositol phosphates were eluted sequentially according to Berridge [16] as described [17]. In some experiments inositol phosphates were separated by anion exchange HPLC on a Partisil 10 SAX column (Whatman, UK) with an ammonium formate gradient (0–1.7 M) [18]. The peaks of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were identified with the elution profile of pure standards obtained from Amersham International, UK. The nature of Ins(1,3,4)P_3 was presumed on the basis of its coelution with ATP [19]. A 24 h pretreatment of the cells with TPA reduced the total water-soluble [^{3}H]inositol content of the cells. Since water soluble [^{3}H]inositol and its phosphorylated metabolites were coprocessed in the separation assay, and thus should be subjected to the same losses, the ratios of the phosphorylated compounds relative to total water-soluble [^{3}H]inositol were calculated for each sample.

2.5. *Angiotensin II binding assay*

Approx. 10^8 cells (approx. 20 mg membrane protein) were suspended in 3–5 ml phosphate buffered saline (PBS) and stored at −70°C. The frozen cells were thawed, homogenised in 20 mM $NaHCO_3$ and centrifuged at 30000 × g for 20 min. The pellet was suspended in 50 mM Tris/HCl, pH 7.4 as described [20]. Briefly, aliquots of 200 μl (10 μg protein) were incubated for 60 min at 25°C with ^{125}I-angiotensin II in the presence or absence of cold ligand (0.1–5.0 nM). Nonspecific binding was determined in the presence of 1 μM cold angiotensin II. The incubation was then terminated by adding ice-cold PBS buffer and filtering through Whatman GF/F filters, washing twice. The filters were counted in a gamma-counter. The concentration of binding sites (B_{max}) and equilibrium dissociation constant (K_d) were determined as described [20].

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

$(Ca^{2+})_i$ was measured by using the fluorescent calcium indicator fluo-3. About 2 × 10^7 cells were incubated at 37°C with 15 μM fluo-3/AM in RPMI 1640 for 30 min. 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+}. After additional 30 min at 37°C the cells were washed again and the angiotensin II-induced fluorescence changes of fluo-3-loaded cells were measured by using a Perkin-Elmer MPF-66 spectrofluorophotometer. The excitation wavelength was fixed at 508 nm (± 5 nm) and the emission wavelength at 535 nm (± 7 nm). The fluorescence signals were calibrated at the end of each individual scan essentially as described by Tsien et al. [21]. A modified Bateman function was used to fit an appropriate elimination constant to the experimental data. The model takes into account an exponential behaviour as follows:

$$\text{Intensity} = D_1(2^{-t/\tau_{el}} - 2^{-t/\tau_{in1}}) + D_2(1-2^{-t/\tau_{in2}})$$

D_1;D_2 = maximal responses of first and second calcium channel (relative intensity units)

τ_{el} = fluorescence half-life elimination constant

t = time after angiotensin II addition

τ_{in1}; τ_{in2} = fluorescence half-life rise times of first and second calcium channel

The model is based on the idea that two mechanisms are responsible for the release and reuptake of Ca^{2+}. The first process (first bracket) characterizes the $InsP_3$-induced release of Ca^{2+} (first calcium channel). The second Ca^{2+} channel (second bracket) corresponds to an increase of $(Ca^{2+})i$ by opening calcium channels located in the plasma membrane. Further details will be shown elsewhere (M. Ochsner and J. Pfeilschifter, manuscript in preparation).

2.7. *Contraction studies*

All contraction studies were performed after a single passage of the same primary culture. Cells received fresh medium containing the indicated concentrations of TPA or vehicle 24 h before the experiment. At the start of the experiments mesangial cells were washed twice and then incubated with Hanks' balanced salt solution at 37°C. Phase micrographs of 3–5 cells were taken before (0 min) and at 3 and 5 min after the addition of angiotensin II (10 nM) as described [4]. Measurements of cell surface area were carried out using a Kontron digitizer tablet. A decrease in cell surface area of more than 12% was considered a contraction.

3. RESULTS

Stimulation of mesengial cells by angiotensin II (100 nM) caused a rapid increase of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 values as shown in fig.1. The Ins(1,3,4)P_3 isomer showed a somewhat delayed increase. We have previously shown that a 24 h pretreatment of mesangial cells with 500 nM TPA diminished soluble and particulate protein kinase C activity to values below 10% of that of control cells. Protein kinase C down-regulated mesangial cells displayed an enhanced response towards an angiotensin II challenge as shown in fig.1. The initial rate of accumulation of the two $InsP_3$ isomers and of $InsP_4$ was augmented and remained at elevated levels compared to control cells

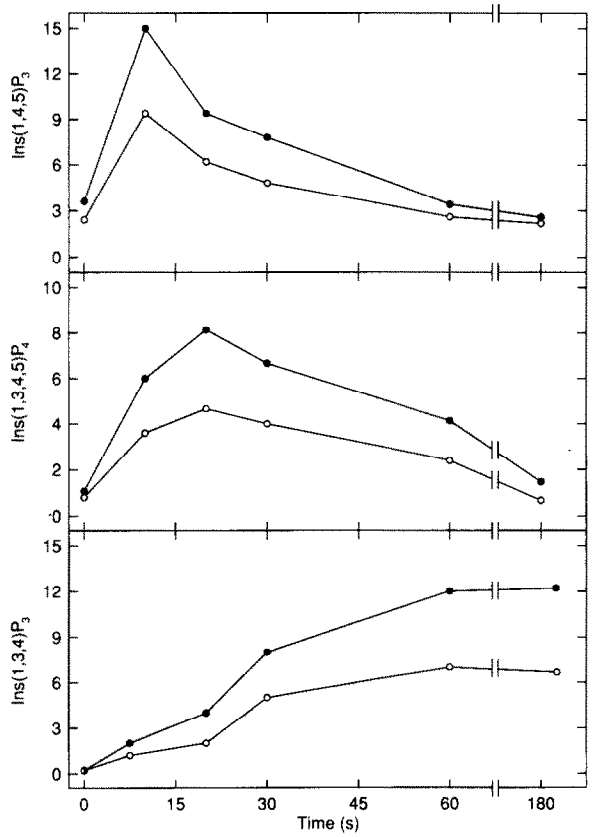

Fig.1. Time-course of angiotensin II-stimulated inositol polyphosphate formation in control cells (○) and cells after 24 h pretreatment with 500 nM TPA (●). Results are expressed as 10^3 × (radioactivity in inositol polyphosphates/radioactivity of total water-soluble inositol) and are means of 5 experiments; the SE ranges from 3 to 10%.

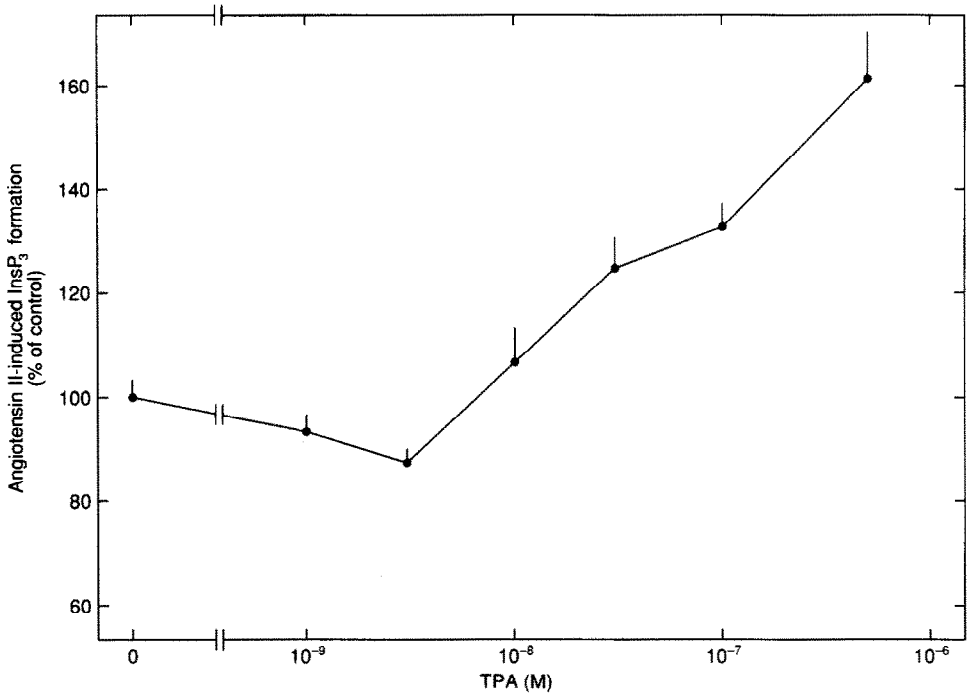

Fig.2. Dose-dependency of 24 h TPA treatment on angiotensin II-stimulated $InsP_3$ formation. Results are expressed as % of control, means ± SE for 5 experiments.

for at least 3 min. TPA treatment also increased the basal values of Ins(1,4,5)P_3. The stimulatory effect of TPA was dose-dependent as shown in fig.2. A minimum concentration of 30 nM of TPA was necessary to obtain a significant enhancement of angiotensin II-induced $InsP_3$ generation. To exclude the possibility that TPA-induced enhancement of the angiotensin II effects after 24 h pretreatment was due to an increased density or affinity of receptors on mesangial cells the specific binding of (^{125}I) angiotensin II was determined. A linear Scatchard plot was obtained suggesting one class of receptor sites. The maximal binding sites (4776 fmol/mg protein) and the apparent dissociation constant K_d (0.267 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 (1 μM) for 24 h. Fig.3 shows the fluorescence response to angiotensin II (100 nM) of mesangial cells loaded with fluo-3. The $(Ca^{2+})_i$ in unstimulated cells was calculated to be 165 ± 26 nM, and angiotensin II increased this value by 78 ± 5 nM (mean ± S.D., $n = 11$ or 9, respectively) within 10–15 s. TPA treatment had no effect on basal $(Ca^{2+})_i$ (160 ± 29 nM), but augmented the increase after stimulation with angiotensin II (97 ± 6 nM, mean ± S.D., $n = 12$ or 13, respectively) and delayed the decay in $(Ca^{2+})_i$ observed thereafter (fig.3). A systematic statistical comparison between TPA-treated and control cells demonstrated significant differences with respect to the elimination constant τ_{el}. The value of τ_{el} was calculated to be 9 ± 2 s in control cells and 23 ± 3 s in TPA-treated cells. Cells preincubated with TPA (1 μM) for 24 h showed a significant increase in the percentage of contractions 3 and 5 min after addition of angiotensin II (10 nM) as compared to the respective

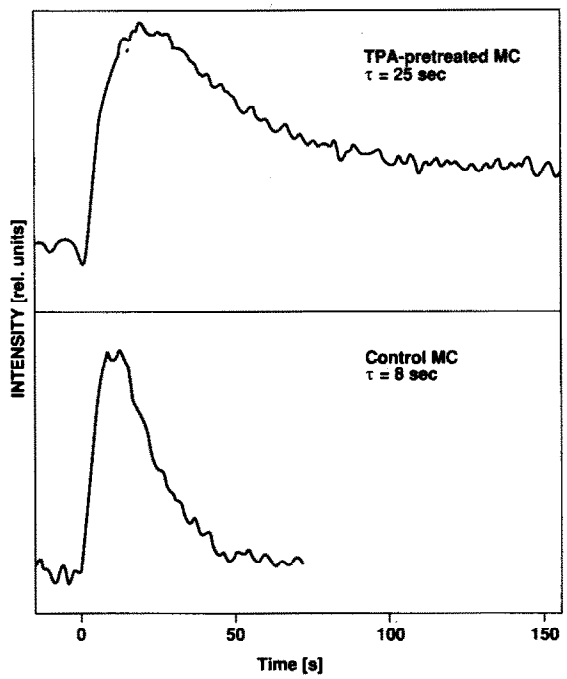

Fig.3. $[Ca^{2+}]_i$ responses to angiotensin II (100 nM) of control and TPA-treated (500 nM, 24 h) mesangial cells (MC) loaded with fluo-3. The x-axis corresponds to the time scale and the y-axis gives the relative intensity of the emitted fluorescence, which can be directly related to $[Ca^{2+}]_i$.

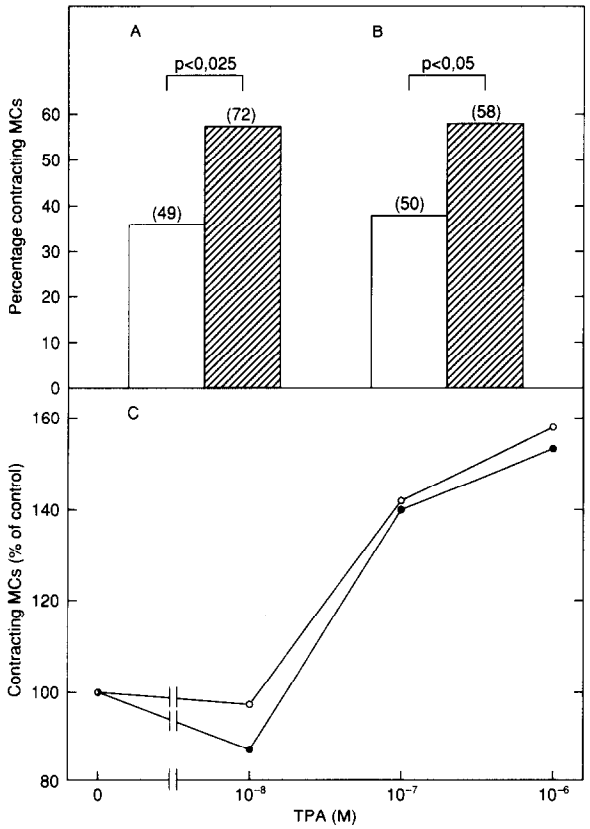

Fig.4. (A), (B) Effect of 24 h TPA (1 μM) (hatched bars) or vehicle (open bars) treatment on angiotensin II-induced contractile activity of mesangial cells (MC). Data are expressed as the percentage of cells contracting 3 min (A), and 5 min (B) after the addition of angiotensin II (10 nM). Numbers in brackets indicate the number of cells evaluated. (C) Dose-dependency of TPA treatment (24 h) on angiotensin II-induced contractile activity 3 min (○) and 5 min (●) after hormone (10 nM) addition.

control group (fig.4A,B). This effect of TPA was dose-dependent, as shown in fig.4C.

4. DISCUSSION

Angiotensin II-, vasopressin- and ATP-induced hydrolysis of phosphoinositides, with the subsequent formation of $InsP_3$ and DG and the rise in cytosolic Ca^{2+}, can be attenuated by short-term activation of protein kinase C in mesangial cells [11–13]. These observations suggest that DG formation during hormone receptor activation may feedback to terminate phospholipase C-mediated $InsP_3$ formation and calcium mobilization. Furthermore, such a modulation of signal transduction in calcium-mobilizing cells by protein kinase C has been proposed to be involved in angiotensin II-induced homologous desensitization in mesangial cells [5]. Inhibitors of protein kinase C, such as H-7, sphingosine and cytotoxin I, augmented angiotensin II-stimulated $InsP_3$ formation [5], thus suggesting that blockage of protein kinase C results in an increased responsiveness of the inositol lipid signalling pathway to hormone stimulation. This study therefore was aimed to elucidate the angiotensin II responses in mesangial cells after protein kinase C down-regulation. We report that loss of protein kinase C resulted in a potentiation of angiotensin II-stimulated $InsP_3$ formation and Ca^{2+} mobilization. Enhanced phosphoinositide hydrolysis after long-term TPA treatment was recently reported for ATP-stimulated mesangial cells [13]. Furthermore, down-regulation of protein kinase C in mesangial cells caused the calcium response to angiotensin II to increase in magnitude and to become much more persistent, in a way similar to what has been reported for A431 cells and vascular smooth muscle cells in response to epidermal growth factor, vasopressin or angiotensin II, respectively [22–25]. Little is known about the precise molecular mechanism responsible for the negative feedback inhibition exerted by protein kinase C. The enhanced responsiveness to angiotensin II in protein kinase C down-regulated mesangial cells is not accompanied by an alteration in either the number of cellular receptors for angiotensin II or in their affinity. It appears that protein kinase C alters angiotensin II-induced generation of $InsP_3$ by inhibiting the signal transmission via the coupling G-protein. It has been reported that short-term pretreatment of washed membranes from glomerular mesangial cells [25] with TPA does not affect GTPγS-induced $InsP_3$ formation. However, TPA pretreatment strongly decreases the synergistic effect of angiotension II on GTPγS-induced $InsP_3$ accumulation, thus suggesting that the coupling between the receptor and the G-protein might have been impaired. There may also be alternative target sites for protein kinase C. The marked prolongation of the Ca^{2+} elevation in protein kinase C down-regulated cells underscores the probable action of the kinase in modulating several key-steps in the signalling pathway from the angiotensin II receptor to cell contraction. This may include modulation of Ca^{2+} homeostatic mechanisms such as the activation of the plasma membrane or endoplasmic reticulum Ca^{2+}-ATPase [26,27] or inhibition of agonist-stimulated Ca^{2+} influx [28]. Further studies will be necessary to elucidate whether such a mode of action of protein kinase C also applies for mesangial cells. The effect of protein kinase C down-regulation in potentiating the contractile response of mesangial cells to a challenge with angiotensin II was unexpected. There is good evidence that protein kinase C contributes to the tonic phase of smooth muscle contraction [29] and it has also been demonstrated that TPA causes a slow contraction of mesangial cells [12]. However, our results show that $(Ca^{2+})_i$ is the major determinant of angiotensin II-induced contraction. The contribution of protein kinase C is an inhibitory one, leading to a rapid desensitization of the angiotensin II-stimulated signalling pathways. This might also explain why angiotensin II fails to pro-

duce a sustained contraction of mesangial cells. However, we cannot exclude the possibility that the small amount of residual protein kinase C activity remaining in the cells following down-regulation might be responsible for cell contraction. It is well documented that several subspecies of protein kinase C exist [30] with different susceptibilities for TPA-induced down-regulation [31]. Further studies will be necessary to characterize the different species of protein kinase C present in mesangial cells and to appoint them to specific cellular functions.

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