Down-regulation of protein kinase C potentiates angiotensin II-stimulated polyphosphoinositide hydrolysis in vascular smooth-muscle cells

Josef PFEILSCHIFTER,* Martin OCHSNER, Steven WHITEBREAD and Marc DE GASPARO
Ciba-Geigy Ltd., Research Department, Pharmaceuticals Division, CH-4002 Basel, Switzerland

In smooth-muscle cells (SMC) isolated from rat aorta, angiotensin II stimulates a phospholipase C with subsequent formation of inositol trisphosphate (InsP_3). Short-term (10 min) pretreatment of SMC with 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 100 nM) decreases the angiotensin II-induced InsP_3 formation. However, this inhibition is not observed after incubating the cells for 2 h with TPA. Longer-term pretreatments even lead to an enhanced generation of InsP_3 . This increased response to angiotensin II occurs without a significant change in the receptor number or K_d value of angiotensin II binding to the cells. The biologically inactive phorbol ester 4 α -phorbol 12,13-didecanoate was without effect on angiotensin II-stimulated InsP_3 generation, irrespective of the time of preincubation. In parallel with this potentiation of angiotensin II-induced generation of InsP_3 by TPA, a down-regulation of protein kinase C activity is observed. A 24 h pretreatment of SMC with TPA decreases protein kinase C activity to less than 10% of that of control cells. Longer-term pretreatment also increases the angiotensin II-induced release of Ca^{2+} and delays the decay of the transient Ca^{2+} increase. All these data suggest that protein kinase C exerts a negative feedback control on angiotensin II-stimulated polyphosphoinositide turnover, and that protein kinase C is an important factor in limiting the production of InsP_3 in stimulated cells.

INTRODUCTION

Desensitization is a widespread regulatory phenomenon in which exposure of a cell to a variety of hormones acting by way of membrane-bound receptors results in a decrease in the number of such receptors or the magnitude of the physiological response induced by the hormone (Sibley & Lefkowitz, 1985). For those cell types in which several hormones act through a common mechanism, desensitization is considered homologous if the diminished biological response is selective for one agonist and heterologous when one agonist induces refractoriness to several hormones.

Angiotensin II is a potent vasoconstrictor in many vascular beds (Gross, 1971; Regoli *et al.*, 1974). In most vessels, however, the hormone fails to produce a sustained contraction, reflecting a specific homologous desensitization phenomenon (McGiff & Itskovits, 1964; Aiken & Vane, 1972). The direct action of angiotensin II on vascular SMC results in a rapid formation of InsP_3 and diacylglycerol, which are concomitantly generated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Smith *et al.*, 1984; Capponi *et al.*, 1985; Alexander *et al.*, 1985). InsP_3 mobilizes Ca^{2+} from endoplasmic reticulum, whereas diacylglycerol activates protein kinase C (Berridge, 1984). These biochemical signals then interact to cause contraction of SMC (Sommerville & Hartshorne, 1986). Angiotensin II- and vasopressin-induced generation of InsP_3 and subsequent Ca^{2+} mobilization have been shown to be decreased or abolished by short-term preincubation with tumour-promoting phorbol esters

(Brock *et al.*, 1985; Caramelo *et al.*, 1988). Similar results have been obtained in different cell types, suggesting a general role for protein kinase C as a negative feedback regulator of cell function (MacIntyre *et al.*, 1985; Sagi-Eisenberg *et al.*, 1985; Naccache *et al.*, 1985; Lynch *et al.*, 1985; Drummond, 1985; Pfeilschifter, 1986).

To elucidate the contribution of protein kinase C to cellular function, two further approaches have been chosen. One approach is the use of inhibitors of protein kinase C, which is limited, however, by the partial selectivity of drugs (Hidaka *et al.*, 1984; Hannun *et al.*, 1986; Tamaoki *et al.*, 1986; Kuo *et al.*, 1983). Alternatively, long-term exposure of cells to phorbol esters results in a down-regulation of protein kinase C and disappearance of phorbol ester responsiveness (Rodriguez-Pena & Rozengurt, 1984; Ballester & Rosen, 1985). In the present paper we report 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 in SMC. These data conform well with the role of protein kinase C as a negative feedback regulator of the inositol lipid signalling pathway in these cells.

MATERIALS AND METHODS

Chemicals

Angiotensin II, TPA and 4 α -phorbol 12,13-didecanoate were from Sigma, St. Louis, MO, U.S.A.; angiotensin II was also obtained from Bachem, Bubendorf, Switzerland; *myo*-[2- ^3H]inositol was purchased

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SMC, smooth-muscle cells; DMEM, Dulbecco's modified Eagle's medium; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concn.; EGF, epidermal growth factor.

* To whom correspondence should be addressed.

from Amersham International, Amersham, Bucks., U.K.; [*tyrosyl*-¹²⁵I]angiotensin II was from Anawa, Wangen, Switzerland; quin 2/AM and ionomycin were from Calbiochem, Lucerne, Switzerland; all cell-culture nutrients were from Boehringer, Mannheim, Germany; all other chemicals used were from Merck, Darmstadt, Germany.

Cell culture

Cultures of SMC from rat aorta were prepared by the explant technique of Ross (1971) as described by Pfeilschifter & Rüegg (1987). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) fetal-calf serum, glutamine and antibiotics. Clones of SMC were characterized morphologically by phase-contrast microscopy and positive staining for the intermediate-filament desmin, which is considered to be specific for myogenic cells (Travo *et al.*, 1982).

Preparation of soluble and particulate fractions

Confluent SMC in 100 mm-diam. dishes were washed twice and incubated with 10 ml of DMEM containing 2% (v/v) fetal-calf serum with or without the indicated concentration of phorbol ester. After the indicated time periods, the dishes were put on ice and the medium was aspirated and replaced by 3.2 ml of homogenization buffer (20 mM-Tris/HCl, pH 7.5, 5 mM-EGTA, 250 mM-sucrose, 0.1 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride). Cells were scraped off with a rubber policeman and sonicated on ice for 3 × 10 s (Branson sonifier, setting 4). After centrifugation at 100 000 *g* at 4 °C for 60 min, the supernatants (cytosolic fractions) were removed and kept on ice until assayed for protein kinase C activity. The pellets were resuspended in the original volume of homogenization buffer containing 1% Nonidet P40 by sonicating for 2 × 15 s, and incubated on ice for 1 h, followed by centrifugation at 100 000 *g* at 4 °C for 60 min. The supernatants were removed and used as 'particulate fraction' in the protein kinase C assay. Protein determination was performed by the method of Bradford (1976).

Assay for protein kinase C

Protein kinase C activity was assayed by measuring the ³²P incorporation from [γ -³²P]ATP into lysine-rich histone from calf thymus (type IIIS; Sigma) in the presence of Ca²⁺, phosphatidylserine and diolein, as described elsewhere (Pfeilschifter, 1988).

Cell labelling

Confluent SMC in 35 mm-diam. dishes were labelled for 48 h with *myo*-[2-³H]inositol (10 μ Ci/ml) in DMEM free of inositol, containing 2% dialysed fetal-calf serum. Phorbol esters or vehicle were added, when not otherwise indicated, for the last 24 h of the labelling period.

Extraction and determination of inositol phosphates

After the prelabelling period, the medium was removed and the cells were rinsed several times to remove free [³H]inositol and incubated for an additional 1 h in fresh medium. After this procedure SMC were incubated in 1 ml of DMEM with or without angiotensin II for the indicated time periods. Thereafter the reaction was terminated 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, Germany). Free inositol and the inositol phosphates were eluted sequentially in accordance with Berridge (1983), as described by Pfeilschifter *et al.* (1986). In some experiments inositol phosphates were separated by anion-exchange h.p.l.c. on a Partisil 10 SAX column (Whatman, Maidstone, Kent, U.K.) with an ammonium formate gradient (0–1.7 M), by the method described by Irvine *et al.* (1986). The peaks of inositol, Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were identified with the elution profile of pure standards obtained from Amersham International. The nature of Ins(1,3,4)P₃ was presumed on the basis of its co-elution with ATP (Irvine *et al.*, 1985). A 24 h pretreatment of SMC with TPA decreased the total water-soluble [³H]inositol content of the cells (as determined by the radioactivity eluted with the first wash of the Dowex columns with distilled water). Since water-soluble [³H]inositol and its phosphorylated metabolites were co-processed in the separation assay, and thus should be subject to the same losses, the ratios of the phosphorylated compounds relative to total water-soluble [³H]inositol were calculated for each sample.

Angiotensin II binding assay

Approx. 10⁸ cells (approx. 20 mg of membrane protein) were suspended in 3–5 ml of phosphate-buffered saline (PBS; 137 mM-NaCl/2.7 mM-KCl/8 mM-Na₂HPO₄/1.5 mM-KH₂PO₄) and stored at –70 °C. The frozen cells were thawed, homogenized (Polytron, Kinematica, Lucerne, Switzerland) in 20 mM-NaHCO₃ and centrifuged at 30 000 *g* for 20 min. The pellet was suspended in 50 mM-Tris/HCl, pH 7.4, containing 125 mM-NaCl, 6.5 mM-MgCl₂, 1 mM-EDTA, 2 mg of bovine serum albumin/ml and a cocktail of peptidase inhibitors: antipain, phosphoramidon, leupeptin, pepstatin A, bestatin and amastatin, each at a concentration of 1 μ g/ml. Portions (200 μ l; 10 μ g of protein) were incubated for 60 min at 25 °C with ¹²⁵I-angiotensin II in the presence or absence of unlabelled ligand (0.1–5.0 nM). Non-specific binding was determined in the presence of 1 μ M unlabelled angiotensin II. The incubation was then terminated by adding ice-cold PBS and filtering through Whatman GF/F filters, with two washes. The incubation and filtration were performed automatically with a Filter-Prep 101 (Ismatec, Zürich, Switzerland). The filters were counted for radioactivity in a gamma-counter. The concentration of binding sites (*B*_{max}) and equilibrium dissociation constant (*K*_d) were determined by using the LIGAND program (Munson & Rodbard, 1980).

Measurement of [Ca²⁺]_i

[Ca²⁺]_i was measured by using the fluorescent Ca²⁺ indicator quin 2 as described by Pfeilschifter & Bauer (1986). About 2 × 10⁷ cells were incubated with 25 μ M-quin 2/AM in DMEM for 60 min. After the incubation period, samples of 1 × 10⁶ cells were washed twice and resuspended in Hepes-buffered saline containing 1.3 mM-Ca²⁺. The angiotensin II-induced fluorescence changes of quin 2-loaded cells were measured at 37 °C by using a Perkin-Elmer MPF-66 spectrofluorophotometer. The excitation wavelength was fixed at 340 nm (± 10 nm) and the emission wavelength at 500 nm (± 10 nm). The

Fig. 1. Time course of TPA effects on basal and angiotensin II-stimulated formation of InsP_3

SMC were prelabelled with $\text{myo-}^3\text{H}$ inositol, treated with TPA (500 nM) (●, ▲) or vehicle (○, △) for the indicated time periods and then exposed to angiotensin II (100 nM) (△, ▲) or vehicle (○, ●) for 30 s. InsP_3 was separated on Dowex columns as described in the Materials and methods section. Results are expressed as radioactivity in ^3H InsP_3 in relation to total water-soluble ^3H inositol. Each value represents the mean $\pm$ S.E.M. of five experiments.

fluorescence signals were calibrated at the end of each individual scan essentially as described by Tsien *et al.* (1982).

A modified Bateman function was used to fit the experimental data and calculate the intracellular Ca^{2+} release and sequestration. The model takes into account an exponential behaviour as follows:

$$\text{Intensity} = D \cdot (2^{-(t-\text{TS})/\tau_{\text{el}}} - 2^{-(t-\text{TS})/\tau_{\text{in}}})$$

where D = maximal response (relative intensity units), τ_{el} = fluorescence half-life elimination constant, TS = moment of angiotensin II addition, and τ_{in} = fluorescence rise time constant.

A systematic comparison between TPA-pretreated and control cells proved that the release of Ca^{2+} characterized by D and τ_{in} (approx. 3 s) is not significantly changed, whereas the elimination of Ca^{2+} from the cytoplasm (τ_{el}) is strongly affected.

RESULTS

Time course of TPA effects on angiotensin II-stimulated InsP_3 formation

Stimulation of SMC prelabelled with ^3H inositol by angiotensin II (100 nM) for 30 s causes marked increases in formation of InsP_1 (+195%), InsP_2 (+925%) as well as InsP_3 (+470%), consistent with the data published in the literature (Smith *et al.*, 1984; Alexander *et al.*, 1985). Preincubation of SMC with TPA (500 nM) for 10 min has no significant effect on the basal InsP_3 values, but significantly attenuates angiotensin II-induced formation of InsP_3 (Fig. 1). These data agree well with the report by Brock *et al.* (1985). However, this inhibition is no longer observed after incubating SMC for 2 h with TPA

(500 nM), as shown in Fig. 1. In contrast, longer-term pretreatments with TPA even lead to an enhanced basal, as well as angiotensin II-stimulated, formation of InsP_3 (Fig. 1). The non-tumour-promoting 4 α -phorbol 12,13-didecanoate has no effect on angiotensin II-induced InsP_3 formation, irrespective of the time of preincubation (results not shown). Although short-term incubation of SMC with TPA has no effect on total water-soluble ^3H inositol content of the cells, 24 h pretreatment with TPA decreases this quantity by approx. 60–70%. This may be due to a decreased uptake of inositol in protein kinase C-down-regulated cells. To compensate for this difference, we express the amounts of ^3H inositol polyphosphates as percentages of total water-soluble ^3H inositol.

Time course of TPA-induced protein kinase C down-regulation

An important aspect of the activation process of protein kinase C appears to be the translocation of the enzyme from the cytosolic compartment to the plasma membrane (Nishizuka, 1986). Fig. 2 shows the time course of this translocation of protein kinase C in SMC. Upon addition of TPA, there is a rapid increase in the membrane-associated protein kinase C activity, accompanied by a corresponding decrease of the cytosolic activity. With increasing time of the TPA treatment, a loss of protein kinase C activity can be demonstrated in SMC (Fig. 2), as has been shown for other cell systems (Rodriguez-Pena & Rozengurt, 1984; Collins & Rozengurt, 1984; Blackshear *et al.*, 1985). These changes are probably due to an increased rate of degradation of protein kinase C in the phorbol-ester-treated cells (Ballester & Rosen, 1985; Young *et al.*, 1987). A 24 h treatment of SMC with TPA (500 nM) results in a down-regulation of protein kinase C activity to less than 10% of that of control cells (Fig. 2).

Fig. 2. Time course of protein kinase C activity in soluble and particulate fraction of TPA-stimulated SMC

SMC were stimulated for the indicated time periods with TPA (500 nM). Thereafter the cells were scraped off, and protein kinase C activity was determined in the soluble (●) and particulate (○) fractions of SMC as outlined in the Materials and methods section. Results are means from a representative experiment done in duplicate.

Fig. 3. Dose-dependency of TPA effects after short-term and long-term pretreatment on angiotensin II-induced InsP_3 formation

SMC were prelabelled with *myo*-[^3H]inositol, pre-treated with the indicated concentrations of TPA for 10 min (○) or 24 h (●), respectively, and then stimulated with angiotensin II (100 nM) for 20 s. InsP_3 was separated on Dowex columns as outlined in the Materials and methods section. Results are expressed as % of control, means $\pm$ S.E.M. for five experiments. The average c.p.m./mg of protein in angiotensin II-stimulated control cells at 20 s was 6720 ± 530 for InsP_3 , and accounted for $0.42 \pm 0.03\%$ of radioactivity in total water-soluble [^3H]inositol.

Dose-dependency of TPA effects on angiotensin II-stimulated InsP_3 formation

The inhibitory effect of short-term pretreatment with TPA is dose-dependent, as shown in Fig. 3. Significant effects are already obtained with 10 nM-TPA, well in accordance with previously published data from Brock *et al.* (1985). In contrast, the stimulatory effect of TPA required much higher doses (Fig. 3). At least 100 nM-TPA was necessary to obtain a significant enhancement of angiotensin II-induced InsP_3 generation, indicating that a complete and full activation of the cellular protein kinase C pool is necessary in order to establish this effect.

Time course of angiotensin II-stimulated inositol phosphate generation in protein kinase C-down-regulated SMC

In control SMC, angiotensin II (100 nM) stimulated a rapid increase in $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, as shown in Fig. 4. The $\text{Ins}(1,3,4)\text{P}_3$ isomer showed a somewhat delayed increase, as expected from the sequential phosphorylation and dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ respectively (Berridge, 1987; Irvine *et al.*, 1988). The protein kinase C-down-regulated SMC show an enhanced response towards an angiotensin II challenge, as shown in Fig. 4. The initial rates of accumulation of the two InsP_3 isomers and of InsP_4 are increased, and remain elevated compared with control cells for at least 5 min. Long-term treatment with TPA also increases the basal values of inositol phosphates in SMC. This increased responsiveness of SMC to stimulation with angiotensin II extends over the whole

Fig. 4. Time course of angiotensin II-stimulated inositol polyphosphate formation in control cells and cells after long-term pretreatment with TPA

SMC were prelabelled with *myo*-[^3H]inositol, pretreated with TPA (500 nM) (●) or vehicle (○) for 24 h and then stimulated with angiotensin II (100 nM) for the indicated time periods. $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were separated by anion-exchange h.p.l.c. as described in the Materials and methods section. Results are expressed as $10^3 \times$ (radioactivity in [^3H]inositol polyphosphates / radioactivity of total water-soluble [^3H]inositol). Each value represents the mean of 3 experiments; the S.E.M. ranges from 6 to 15%.

dose-response range, as shown in Fig. 5. The potentiation of angiotensin II-induced formation of InsP_3 occurs without a change in the ED_{50} for the hormone.

Effect of protein kinase C down-regulation on angiotensin II-stimulated Ca^{2+} mobilization

As $\text{Ins}(1,4,5)\text{P}_3$ is the second messenger involved in the mobilization of Ca^{2+} from intracellular stores (Berridge & Irvine, 1984), one would expect an amplified mobilization of intracellular Ca^{2+} in protein kinase C-down-regulated SMC. Fig. 6 shows that angiotensin II induces a rapid and pronounced increase in $[\text{Ca}^{2+}]_i$ in SMC loaded with quin 2. The $[\text{Ca}^{2+}]_i$ in unstimulated cells was calculated to be 170 ± 23 nM, and angiotensin II increases this value to a maximum of 372 ± 77 nM (mean $\pm$ S.E.M., $n = 4$) within 10–15 s. Down-regulation of protein kinase C by 24 h treatment with TPA (500 nM) had no effect on basal $[\text{Ca}^{2+}]_i$, but increased the peak value after stimu-

Fig. 5. Dose-dependency of angiotensin II-stimulated InsP_3 formation in control cells and cells after long-term pretreatment with TPA

SMC were prelabelled with $\text{myo-}[^3\text{H}]\text{inositol}$, pre-treated with TPA (500 nM) (●) or vehicle (○) for 24 h, and then stimulated with the indicated concentrations of angiotensin II for 30 s. InsP_3 was separated on Dowex columns as described in the Materials and methods section. Results are expressed as radioactivity in $[^3\text{H}]\text{InsP}_3$ in relation to total water-soluble $[^3\text{H}]\text{inositol}$. Each value represents the mean $\pm$ S.E.M. of five experiments.

lation of SMC with angiotensin II and delayed the decay in $[\text{Ca}^{2+}]_i$ observed thereafter (Fig. 6). The fact that down-regulation of protein kinase C increases basal values of InsP_3 without altering $[\text{Ca}^{2+}]_i$, as measured with quin 2, might be explained by the buffering capacity of quin 2, which makes it unable to resolve small increases in $[\text{Ca}^{2+}]_i$.

Effects of TPA on angiotensin II binding to SMC

It has been reported by Brock *et al.* (1985) that short-term pretreatment of SMC with TPA does not affect angiotensin II-binding characteristics. To exclude the possibility that TPA-induced enhancement of the angiotensin II effects after long-term pretreatment is due to an

Table 1. Effect of TPA pretreatment of SMC on angiotensin II-receptor binding constants

SMC were pretreated with the indicated concentration of TPA for 24 h, and angiotensin II binding assay was performed as described in the Materials and methods section. Results represent means from three (control, 500 nM) or two (10 and 100 nM) separate experiments performed in duplicate.

Addition	K_d (nM)	S.E. (%)	B_{max} (fmol/mg)	S.E. (%)
Control	0.365	4.57	1183.3	2.09
TPA (10 nM)	0.414	15.98	1189.7	8.05
TPA (100 nM)	0.335	6.27	1136.6	2.77
TPA (500 nM)	0.441	8.23	1130.4	4.15

Fig. 6. $[\text{Ca}^{2+}]_i$ in SMC as indicated by quin 2 fluorescence

Fluorescence response to angiotensin II of SMC loaded with quin-2/AM. The upper spectrum was recorded after pretreating the cells with TPA (500 nM, 24 h), whereas the lower spectrum shows the response in control cells. The ordinate gives the relative intensity of the emitted fluorescence, which can be directly related to $[\text{Ca}^{2+}]_i$, as shown elsewhere (Berthon *et al.*, 1984). Despite the different fluorescence levels in the two samples, the Ca^{2+} levels are the same.

increased density or affinity of receptors on SMC, we determined the specific binding of ^{125}I -angiotensin II. A linear Scatchard plot was obtained, corresponding to one class of receptor sites. 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 (10–500 nM) for 24 h (Table 1).

DISCUSSION

Angiotensin II- and vasopressin-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate, with the subsequent formation of InsP_3 and diacylglycerol and the rise in cytosolic Ca^{2+} , can be attenuated by short-term activation of protein kinase C in SMC (Brock *et al.*, 1985; Caramelo *et al.*, 1988). These observations suggest that diacylglycerol formation during angiotensin II- or vasopressin-receptor activation may feed-back to terminate phospholipase C-mediated InsP_3 formation and Ca^{2+} mobilization. Furthermore, such a modulation of signal transduction in Ca^{2+} -mobilizing cells by protein kinase C has been proposed to be involved in homologous desensitization in SMC-derived DDT₁ MF-2 cells (Leeb-Lundberg *et al.*, 1985), in platelet-activating-factor-induced homologous and heterologous desensitization in SMC (Schwertschlag & Whorton, 1988), in angiotensin II-induced homologous desensitization in mesangial cells (Pfeilschifter, 1988), and in angiotensin II-, vasopressin- and EGF-induced desensitization in a continuous liver epithelial-cell line (Hepler *et al.*, 1988).

To investigate further the role of protein kinase C in various cellular functions, inhibitors of protein kinase C, such as 1-(5-isoquinoline sulphonyl)-2-methylpiperazine (H-7) (Hidaka *et al.*, 1984), sphingosine (Hannun *et al.*, 1986), staurosporine (Tamaoki *et al.*, 1986) and others have been employed. Caramelo *et al.* (1988) have shown

that H-7 partially reversed the TPA effects on both the angiotensin II- and vasopressin-induced cytosolic Ca^{2+} increase in SMC. Furthermore, in mesangial cells, H-7, sphingosine and cytotoxin I enhanced angiotensin II-stimulated InsP_3 formation (Pfeilschifter, 1988). The last report agrees well with the idea that blockage of protein kinase C would result in an increased responsiveness of the inositol-lipid signalling pathway to hormone stimulation. However, as none of the compounds used is absolutely specific for protein kinase C, the implication of other types of phosphorylation cannot be ruled out.

In an alternative approach, we have characterized the angiotensin II-induced InsP_3 generation under conditions where protein kinase C has been down-regulated by long-term pretreatment of SMC with the phorbol ester TPA. We report that loss of protein kinase C results in an increased responsiveness of the inositol-lipid signalling cascade to angiotensin II stimulation in SMC. Our results agree well with the data of Brown *et al.* (1987), showing that bombesin-stimulated polyphosphoinositide hydrolysis in Swiss-mouse 3T3 cells is also elevated after long-term pretreatment with TPA. Similar results were obtained by Hepler *et al.* (1988). These authors demonstrated that long-term pretreatment of a continuous liver epithelial-cell line (WB cells) with phorbol ester resulted in a marked sensitization of the phosphoinositide signalling pathway to angiotensin II and EGF stimulation. Furthermore, Pandiella *et al.* (1987) reported that down-regulation of protein kinase C in A431 epidermoid carcinoma cells caused the Ca^{2+} response to EGF to increase in magnitude and to become much more persistent. Wahl & Carpenter (1988) reported that tamoxifen and sphingosine, both potent inhibitors of protein kinase C activity, potentiate the capacity of EGF to stimulate formation of InsP_3 in A431 cells. Interestingly, chronic exposure of A431 cells to TPA results in an enhanced responsiveness to EGF-induced InsP_3 formation.

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 SMC is not accompanied by an alteration either in the number of cellular receptors for angiotensin II or in their affinity. It appears that protein kinase C interferes with angiotensin II-stimulated phosphoinositide hydrolysis in SMC at a post-receptor site, but before the formation of InsP_3 by phospholipase C. A possible target site for protein kinase C-mediated phosphorylation may involve the G-protein that couples angiotensin II receptors to phospholipase C. Katada *et al.* (1985) have demonstrated that protein kinase C is able to phosphorylate the inhibitory guanine-nucleotide-binding regulatory component of adenylate cyclase, thereby suppressing its function. It may be possible that protein kinase C also alters hormone-induced generation of InsP_3 by inhibiting the signal transmission via the coupling G-protein. In support of this idea, other studies demonstrate that short-term pretreatment of washed membranes from glomerular mesangial cells (Pfeilschifter & Bauer, 1987) and from Flow 9000 cells (Lo & Hughes, 1988) with TPA does not affect guanosine 5'-[γ -thio]triphosphate-induced InsP_3 formation. However, TPA pretreatment strongly decreases the potentiating effect of angiotensin II and cholecystokinin, respectively, on guanosine 5'-[γ -thio]triphosphate-induced stimulation of InsP_3 accumulation, suggesting that the coupling between the receptor and

the G-protein might have been impaired. Other reports show that hormone- and guanine-nucleotide-stimulated inositol phosphate formation were attenuated in membranes from astrocytoma cells (Orellana *et al.*, 1987) and polymorphonuclear leukocytes (Smith *et al.*, 1987) after short-term pretreatment with TPA. These authors conclude that the inhibitory action of TPA is exerted on a G-protein and affects its ability to interact with phospholipase C. In contrast with the latter reports, in liver epithelial cells (WB cells) protein kinase C does not regulate agonist-induced InsP_3 generation by altering the capacity of the involved G-protein to activate phospholipase C (Hepler *et al.*, 1988). Obviously, phorbol esters inhibit hormone-stimulated InsP_3 formation in a receptor- and tissue-selective manner. Further studies will be necessary to unveil the exact mechanism of protein kinase C-mediated feedback inhibition of angiotensin II-stimulated InsP_3 formation in SMC.

We gratefully acknowledge the skilful technical assistance of Fiorentina Festa and Barbara Thüning.

REFERENCES

- Aiken, J. W. & Vane, J. R. (1972) *J. Pharmacol. Exp. Ther.* **184**, 678–687
- Alexander, R. W., Brock, T. A., Gimbrone, M. A., Jr. & Rittenhouse, S. E. (1985) *Hypertension* **7**, 447–451
- Ballester, R. & Rosen, O. M. (1985) *J. Biol. Chem.* **260**, 15194–15199
- Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Berthon, B., Binet, A., Mauger, J. P. & Claret, M. (1984) *FEBS Lett.* **167**, 19–24
- Blackshear, P. J., Witters, L. A., Girard, P. R., Kuo, J. F. & Quamo, S. N. (1985) *J. Biol. Chem.* **260**, 13304–13315
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Brock, T. A., Rittenhouse, S. E., Powers, C. W., Ekstein, L. S., Gimbrone, A. A., Jr. & Alexander, R. W. (1985) *J. Biol. Chem.* **260**, 14158–14162
- Brown, K. D., Blakeley, D. M., Hamon, M. H., Laurie, M. S. & Corps, A. N. (1987) *Biochem. J.* **245**, 631–639
- Capponi, A. M., Lew, P. D. & Vallotton, M. B. (1985) *J. Biol. Chem.* **260**, 7836–7842
- Caramelo, C., Tsai, P. & Schrier, R. W. (1988) *Biochem. J.* **254**, 625–629
- Collins, M. K. L. & Rosengurt, E. (1984) *J. Cell Physiol.* **118**, 133–143
- Drummond, A. H. (1985) *Nature (London)* **315**, 752–755
- Gross, F. (1971) *Int. Encycl. Pharmacol.* **72**, 73–286
- Hannun, Y. A., Loomis, C. R., Merrill, A. H. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 12604–12609
- Hepler, J. R., Earp, H. S. & Harden, T. K. (1988) *J. Biol. Chem.* **263**, 7610–7619
- Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) *Biochemistry* **23**, 5036–5041
- Irvine, R. F., Anggard, E. E., Letcher, A. F. & Downes, C. P. (1985) *Biochem. J.* **229**, 505–511
- Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
- Irvine, R. F., Moor, R. M., Pollock, W. K., Smith, P. M. & Wreggett, K. A. (1988) *Philos. Trans. R. Soc. London B* **320**, 281–298
- Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) *Eur. J. Biochem.* **15**, 431–437

- Kuo, J. F., Raynor, R. L., Mazzei, G. J., Schatzman, R. C., Turner, R. S. & Kem, W. R. (1983) *FEBS Lett.* **153**, 183–186
- Leeb-Lundberg, L. M. F., Cotecchia, S., Lomasney, J. W., Debernardis, J. F., Lefkowitz, R. J. & Caron, M. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5651–5655
- Lo, W. W. Y. & Hughes, J. (1988) *Biochem. J.* **251**, 625–630
- Lynch, C. J., Charest, R., Bocckino, S. B., Exton, J. H. & Blackmore, P. F. (1985) *J. Biol. Chem.* **260**, 2844–2851
- MacIntyre, D. E., McNicol, A. & Drummond, A. H. (1985) *FEBS Lett.* **180**, 160–164
- McGiff, J. C. & Itskovits, H. D. (1964). *J. Clin. Invest.* **43**, 2359–2367
- Munson, P. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- Naccache, P. H., Molski, T. F. P., Borgeat, P., White, J. R. & Sha'afi, R. (1985) *J. Biol. Chem.* **260**, 2125–2131
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Orellana, S., Solski, P. A. & Brown, J. H. (1987) *J. Biol. Chem.* **262**, 1638–1643
- Pandiella, A., Vincentini, L. M. & Meldolesi, J. (1987) *Biochem. Biophys. Res. Commun.* **149**, 145–151
- Pfeilschifter, J. (1986) *FEBS Lett.* **203**, 262–266
- Pfeilschifter, J. (1988) *Biochim. Biophys. Acta* **969**, 263–270
- Pfeilschifter, J. & Bauer, C. (1986) *Biochem. J.* **236**, 289–294
- Pfeilschifter, J. & Bauer, C. (1987) *Biochem. J.* **248**, 209–215
- Pfeilschifter, J. & Rüegg, U. T. (1987) *Biochem. J.* **248**, 883–887
- Pfeilschifter, J., Kurtz, A. & Bauer, C. (1986) *Biochem. J.* **234**, 125–130
- Regoli, D., Park, W. K. & Rioux, F. (1974) *Pharmacol. Rev.* **26**, 69–123
- Rodriguez-Pena, A. & Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* **120**, 1053–1059
- Ross, R. (1971) *J. Cell Biol.* **50**, 172–186
- Sagi-Eisenberg, R., Lieman, N. & Pecht, I. (1985) *Nature (London)* **313**, 59–60
- Schwertschlag, U. S. & Whorton, A. R. (1988) *J. Biol. Chem.* **263**, 13791–13796
- Sibley, D. R. & Lefkowitz, R. J. (1985) *Nature (London)* **317**, 124–129
- Smith, C. D., Uhing, R. J. & Snyderman, R. (1987) *J. Biol. Chem.* **262**, 6121–6127
- Smith, J. B., Smith, L., Brown, E. R., Barnes, D., Sabir, M. A., Davis, J. S. & Farese, R. V. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7812–7816
- Sommerville, L. E. & Hartshorne, D. J. (1986) *Cell Calcium* **7**, 353–364
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Travo, P., Weber, K. & Osborn, M. (1982) *Exp. Cell Res.* **139**, 87–54
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–344
- Wahl, M. & Carpenter, G. (1988) *J. Biol. Chem.* **263**, 7581–7590
- Young, S., Parker, P. J., Ullrich, A. & Stabel, S. (1987) *Biochem. J.* **244**, 775–779

Received 25 January 1989/13 April 1989; accepted 2 May 1989