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Activation of protein kinase C by phorbol esters induces DNA synthesis and protein phosphorylations in glomerular mesangial cells

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The tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) is shown to be mitogenic for quiescent glomerular mesangial cells cultured in serum-free conditions. TPA induces DNA synthesis measured by [^{3}H]thymidine incorporation in a dose-dependent manner with an ED_{50} of 7 ng/ml and an optimal response for 50 ng/ml. The phorbol ester action is potentiated by insulin with an increase of the maximal effect from 232 ± 15% for TPA alone to 393 ± 96% for TPA plus insulin. Down-regulation of protein kinase C by prolonged exposure to TPA completely abolishes the mitogenic effect of the phorbol ester. Using a highly resolutive 2D electrophoresis, we have shown that TPA is able to stimulate the phosphorylation of 2 major proteins of M_r 80000, pI 4.5 (termed 80K) and M_r 28000, pI 5.7–5.9 (termed 28K). The 80K protein phosphorylation is time- and dose-dependent with an ED_{50} of 8 ng/ml TPA. Exposure of mesangial cells to heat-shock induces synthesis of a 28K protein among a set of other proteins suggesting that the 28K protein kinase C substrate belongs to the family of low molecular mass stress proteins. Mitogenic concentrations of TPA and phorbol 12,13-dibutyrate inhibit [^{125}I]epidermal growth factor binding and stimulate the 80K protein phosphorylation with the same order of potency. The inactive tumor-promoter 4α-phorbol was found to be ineffective both on these 2 parameters and on DNA synthesis. These results suggest a positive role for protein kinase C on mesangial cell proliferation and indicate the existence in this cell line of 2 major protein kinase C substrates.

Phorbol ester; DNA synthesis; Protein kinase C; Protein phosphorylation; Mesangial cell

1. INTRODUCTION

Protein kinase C (PKC), which is activated by diacylglycerols and tumor-promoting phorbol esters plays a major role in the control of a variety of cellular processes including differentiation and proliferation [1,2]. While PKC activators have been shown to induce growth arrest of cultured cells like MCF-7 [3] or HL60 [4], activation of the enzyme can, on the contrary, stimulate cell proliferation in various other cell types such as Swiss 3T3 [5] or vascular endothelial cells [6,7]. Proliferation of glomerular mesangial cells is a prominent feature of most types of progressive glomerular diseases leading to renal dysfunction [8]. Mesangial cell mitogenesis can be regulated by various humoral factors [9]. Some of them such as endothelin [10] or arginin vasopressin [11] can act through phospholipase C activation leading to inositol phosphate and diacylglycerol production.

In order to define a positive role of PKC on mesangial cell proliferation, we investigated the effect of tumor-promoting phorbol ester 12-*o*-tetradecanoylphorbol 13-acetate (TPA) on DNA synthesis. We report that TPA induces mesangial cell mitogenesis. This action correlates with the activation of PKC determined by induction of specific phosphorylations and inhibition of [^{125}I]epidermal growth factor binding.

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Abbreviations: TPA, 12-*o*-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGF, epidermal growth factor; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1. *Chemicals*

Phorbol esters were from Sigma. [^{32}P]phosporic acid, [^{125}I]EGF (600 Ci/mmol), [^{3}H]thymidine (60 Ci/mmol) and [^{3}H]leucine (146 Ci/mmol) were purchased from Amersham. Ampholines were from LKB. All other chemicals were of reagent grade.

2.2. *Methods*

2.2.1. Cell culture

Rat mesangial cells were established following culture of freshly isolated glomeruli and cloned as described in [12]. Stock cultures of cloned mesangial cells were maintained at 37°C in RPMI 1640 (Gibco) containing 10% fetal calf serum, 50 u/ml penicillin and 50 μg/ml streptomycin in humidified 5% CO_2/95% air.

Before each experiment, cells were made quiescent by serum starvation for at least 48 h. After this period, no increase in the cell number was observed suggesting cells were in a resting state.

2.2.2. Mitogenesis assay

For measurement of DNA synthesis, quiescent cells were incubated in 2 ml of RPMI containing [^{3}H]thymidine (1 μCi/ml). Mitogens were added and the cells were incubated at 37°C for 40 h. Reactions were terminated by washing cultures in cold PBS and the incorporation of radioactivity into TCA-precipitable material was determined after cells were dissolved in 1 ml of 2% Na_2CO_3, 1% SDS, 0.1 M NaOH.

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2.2.3. Protein phosphorylation

Subconfluent cultures were washed twice in phosphate-free Krebs-Ringer buffer, pH 7.2, containing 20 mM HEPES, 0.1% BSA, 0.2% glucose and incubated for 2 h at 37°C in 1 ml of the same buffer containing 40 μCi [^{32}P]phosphoric acid. Stimuli were then added for various times. After cell washing, TCA-precipitated proteins were dissolved in SDS sample buffer [13] and analyzed by SDS-PAGE as previously described [14] using 10% acrylamide (w/v) slab gels. Samples to be analyzed by 2D gel electrophoresis were solubilized in 0.1 ml of buffer consisting of 30 mM Tris-HCl, pH 7.5, 1% (w/v) SDS, 4% (v/v) glycerol, 1% (v/v) β-mercaptoethanol and 0.002% bromophenol blue. After heating at 100°C for 5 min, the samples were cooled, then adjusted to contain 9.5 M urea, 3.7% (v/v) nonidet-P40 and 2% (w/v) ampholines, in a final volume of 0.3 ml. Proteins were separated using 1% pH 5-7, 0.5% pH 3.5-9.5 and 0.5% pH 2.5-4.5 ampholines in the isoelectric focusing dimension and a 10% (w/v) acrylamide gel in the second dimension.

Phosphorylation of the 80K protein was specifically examined by exploiting its solubility in Triton-X100 and stability in the detergent during heat treatment [15,16]. Culture medium was aspirated and replaced with 0.2 ml of extraction buffer comprising 50 mM Tris-HCl, pH 6.9, 2 mM EGTA, 10 mM sodium fluoride, 5 μg/ml aprotinin, 50 μM phenylmethylsulfonyl fluoride and 0.5% (v/v) Triton X-100. After a 5 min incubation at room temperature, the extracts were removed from the dish, heated at 100°C for 3 min and then allowed to cool. Precipitated proteins were sedimented by centrifugation at 10 000 × g for 2 min. The supernatant was mixed with an equal volume of 2 × SDS sample buffer and phosphoproteins were separated by SDS-PAGE using an 8% acrylamide (w/v) slab gel.

2.2.4. Protein synthesis under stress conditions

Cultures were submitted to heat-shock by incubation at 43°C for various times. After washing, cells were incubated for 1 h at 37°C in 2 ml of leucine-free medium containing 100 μCi of [^{3}H]leucine. At the end of the incubation, cells were washed twice with cold PBS and 10% TCA was added. TCA-precipitated proteins were analyzed by SDS-PAGE and autoradiography.

2.2.5. [^{125}I]*EGF binding assay*

Cultures were washed twice with RPMI and incubated at 37°C in 1 ml of RPMI containing 50 mM *N,N'*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid, pH 7, and factors as indicated. [^{125}I]EGF binding to the cells was then determined during 2 h at 4°C as described [17] using a concentration of 0.1 nM [^{125}I]EGF (110 000 cpm/ng). Non-specific binding was determined as cell-associated radioactivity in the presence of a 250-fold excess of unlabeled EGF.

3. RESULTS AND DISCUSSION

As shown in Table IA, serum-starved mesangial cells responded to TPA with a significant increase of [^{3}H]thymidine incorporation indicating stimulated DNA synthesis. Insulin (5 μg/ml) had no effect by itself but potentiated the action of TPA (from 232 ± 15% to 393 ± 96%) suggesting that maximal mesangial cell proliferation might be triggered by various mitogens acting synergistically. The effect of TPA alone or combined with insulin was dose-dependent, with in both cases an ED_{50} of 7 ng/ml and a maximal response at 50 ng/ml (Fig. 1). The inactive tumor promoter 4α-phorbol which does not activate PKC, was unable to induce [^{3}H]thymidine incorporation. Moreover, addition of 100 ng/ml TPA to serum-starved mesangial cells yielded a 41 ± 2% (mean ± SE, n = 8, $P < 0.001$) increase

Table I

Stimulation of DNA synthesis by TPA in glomerular mesangial cells

A Addition	[^{3}H]Thymidine incorporation (% of control)	
	Mean ± SEM	n
Insulin 5 μg/ml	96 ± 4	6
TPA 100 ng/ml	232 ± 15	6
TPA 100 ng/ml + Insulin 5 μg/ml	393 ± 96	6
α-Pho 100 ng/ml	101 ± 1	3

B Addition	[^{3}H]Thymidine incorporation (% of control)	
	Normal cells	TPA-pretreated cells
TPA 100 ng/ml	192	100
TPA 100 ng/ml + Insulin 5 μg/ml	271	100
FCS 10%	487	480

(A) Quiescent mesangial cells were incubated for 40 h in serum-free RPMI medium containing [^{3}H]thymidine and the indicated concentrations of factors. At the end of incubation, cells were washed and radioactivity was measured as described in section 2. The values represent the mean ± SEM of 3-6 independent experiments. Each experiment was performed in triplicate. (B) Mesangial cells were incubated for 48 h in serum-free RPMI medium containing 100 ng/ml TPA (TPA-pretreated cells) or no addition (normal cells). Then, cells were washed and incubated with [^{3}H]thymidine as (A). The values represent the mean of triplicates from one representative experiment. Similar results were obtained in another experiment.

in cell number after 2 days of exposure, while 4α-phorbol had no effect (data not shown).

Prolonged exposure of cells with phorbol esters is known to down-regulate PKC in mesangial cells [18] as well as in other cell lines [19,20]. As shown in Table IB, pretreatment of mesangial cells for 40 h with TPA completely abolished the further action of TPA on DNA synthesis, while the serum was still effective. Moreover, the potentiative action of insulin was no longer observed in PKC-depleted cells, agreeing with the inability of insulin to promote by itself mesangial cell mitogenesis.

These results clearly indicate that the mitogenic effect of TPA is most mediated by PKC activation. In order

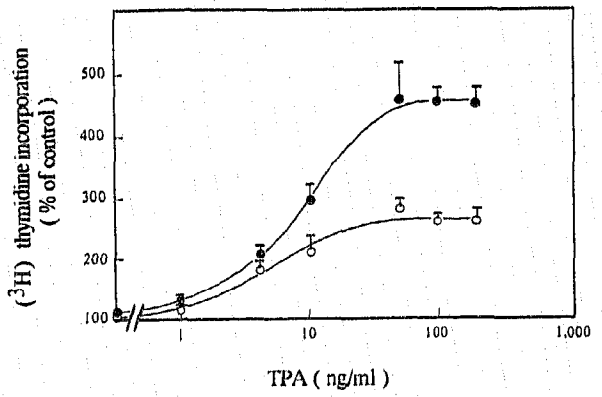

Fig. 1. Effect of various concentrations of TPA on DNA synthesis. [^{3}H]thymidine incorporation was measured after 40 h incubation in medium containing increasing concentrations of TPA in the absence (○) or in the presence (●) of 5 μg/ml insulin. The values represent the mean ± SEM of 4-6 determinations obtained in 2 separate experiments.

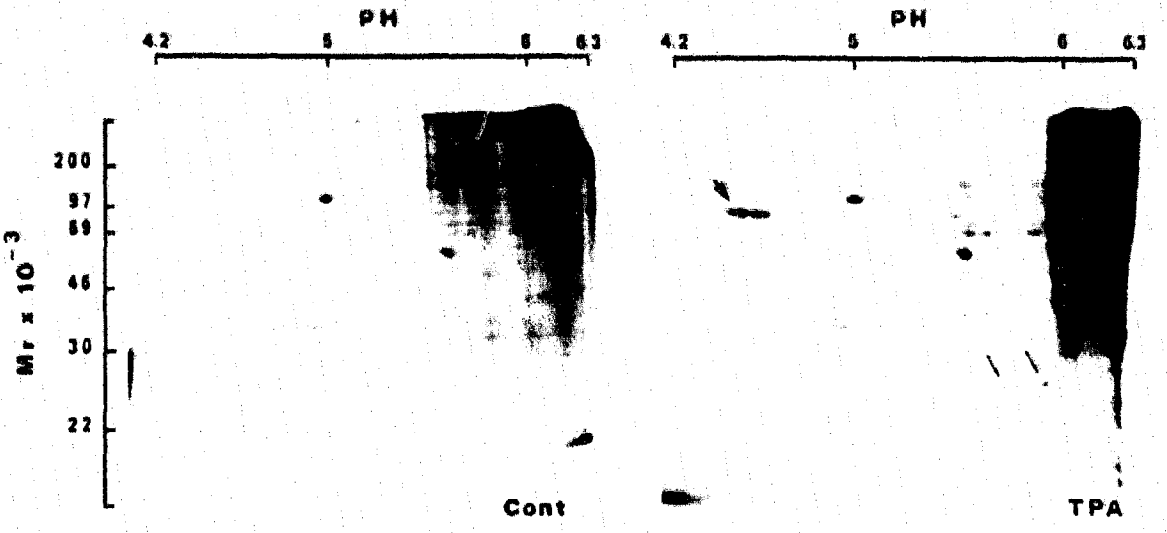

Fig. 2. Effect of TPA on cellular protein phosphorylation in mesangial cells. Mesangial cells labeled with [^{32}P]phosphoric acid for 2 h were treated without (cont) or with 100 ng/ml TPA (TPA) for 30 min. Cellular phosphoproteins were resolved by 2D gel electrophoresis and autoradiography as described in section 2. Thick arrow indicates the position of 80K protein. Thin arrows indicate the position of 28K protein.

to define the early PKC-induced events that may trigger mesangial cell DNA synthesis, we performed a 2D analysis of phosphoproteins from extracts of quiescent mesangial cells labeled with [^{32}P]phosphoric acid.

Figure 2 shows that TPA, at a mitogenic concentration, induced a marked enhancement of a set of phosphoproteins with M_r 78 000–80 000, pI 4.5 (termed 80K), M_r 54 000, pI 5.5 and M_r 28 000, pI 5.7–5.9 (termed 28K).

An 80K phosphoprotein with a pI of 4.7 has been identified as a major and specific substrate of PKC in Swiss 3T3 cells [15,21] and other cultured fibroblasts [16,22]. This protein termed MARCKS (myristoylated alanine rich C kinase substrate) appears to be closely related to the 80K phosphoproteins found in rat and bovine brain [23,24]. They all seem to belong to a family of protein kinase C substrates which does not exhibit significant homology to other known cellular proteins. Phosphorylation of MARCKS protein can be examined by exploiting its solubility in Triton-X100 and stability in the detergent during heat treatment [15,16]. As shown in Fig. 3, using this procedure, we studied the 80K protein phosphorylation induced by TPA as a function of time and concentration. The 80K protein phosphorylation was dose-dependent with an ED_{50} of 8 ng/ml corresponding to the ED_{50} observed for mitogenesis (Fig. 3A, Fig. 1). Figure 3B shows that the 80K protein phosphorylation was rapid (within 2 min) and maximal after 10 min stimulation. These results strongly suggest that the 80K phosphoprotein described in mesangial cells could belong to the MARCKS protein family. This protein provides a specific marker for PKC activation in this cell line.

As shown in Fig. 2, TPA also stimulated the phosphorylation of a 28K protein observed as 2 isoforms of pI 5.7 and 5.9. Similar phosphoproteins have been described in MCF-7 human breast cancer cells [14,25] as well as in endothelial cells [6,26]. In MCF-7 cells, we have demonstrated that phosphorylation of the 28K protein was specific of PKC activation [14]. These proteins have been recently characterized as stress proteins as they were inducible by heat-shock [26] or other stress inducers [27]. We wondered if the 28K phosphoprotein described in mesangial cells could also belong to the family of low molecular mass stress proteins. As shown in Fig. 4, [^{3}H]leucine labeling of mesangial cells incubated at 43°C for various times

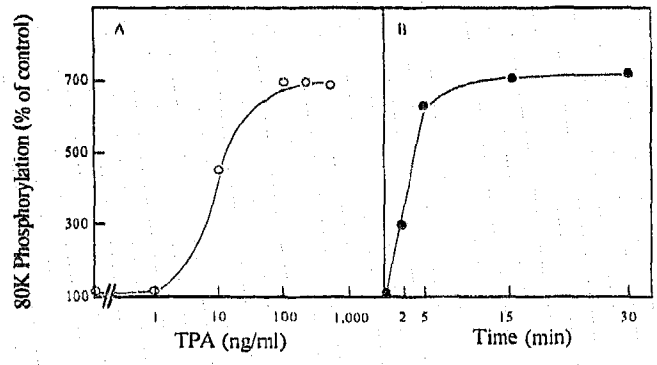

Fig. 3. Time- and dose-dependent stimulation of 80K phosphorylation by TPA. Mesangial cells labeled with [^{32}P]phosphoric acid for 2 h were treated with increasing concentrations of TPA for 15 min (Fig. 3A), or with 100 ng/ml TPA for various times (Fig. 3B). Triton extracts were prepared as described in section 2. The heat-stable proteins were resolved using 1D SDS-PAGE. Autoradiographs were scanned using an LKB densitometer. The data are expressed as percentage of the values obtained without stimulation (control).

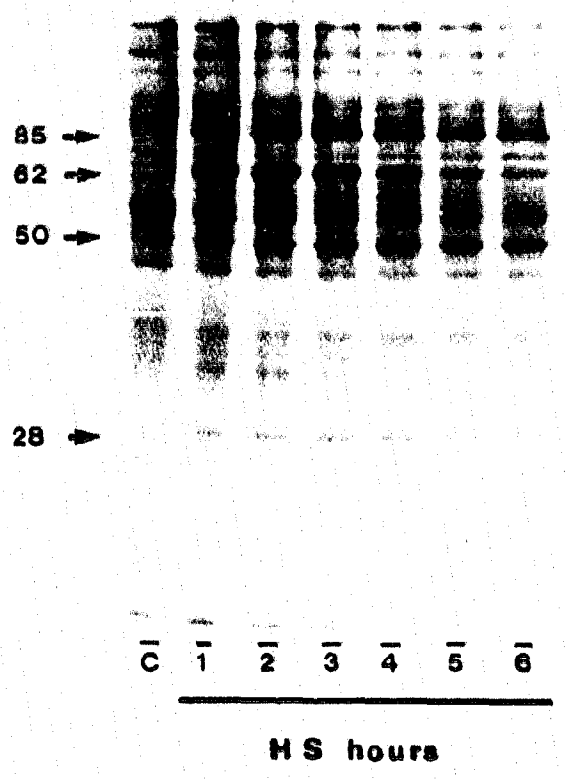

Fig. 4. Effect of heat-shock treatment for various times on mesangial cell protein synthesis. Cells were incubated at 37°C (C) or at 43°C for various times (HS). The cells were then labeled at 37°C for 1 h with [^{3}H]leucine in leucine-free medium. TCA-precipitated proteins were then analysed by SDS-PAGE and autoradiography. Arrows indicate proteins whose synthesis is affected by heat shock.

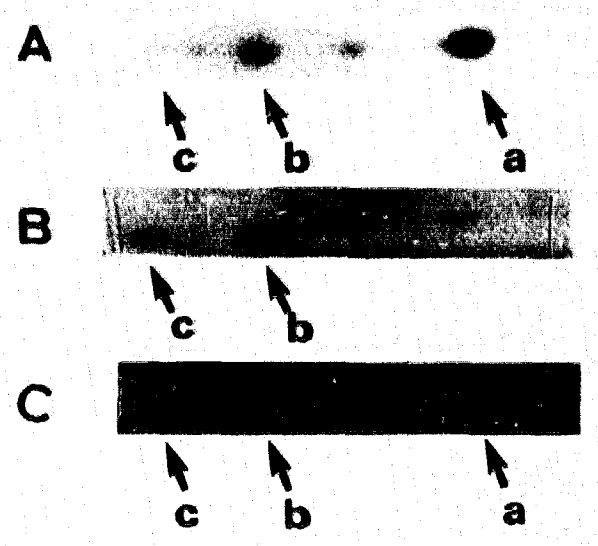

Fig. 5. Two-dimensional gel electrophoresis analysis of the 28K protein synthetized after heat-shock and phosphorylated upon TPA treatment. (A) Mesangial cells were incubated at 43°C for 3 h and then labeled at 37°C for 1 h with [^{3}H]leucine in leucine-free medium. Cellular proteins were resolved by 2D-gel electrophoresis and autoradiography. (B) Mesangial cells were labeled with [^{32}P]phosphoric acid for 2 h and then treated with 100 ng/ml TPA during 15 min. Cellular phosphoproteins were resolved by 2D-gel electrophoresis and autoradiography. (C) Equal volumes of [^{3}H]leucine- and ^{32}P-labeled extracts respectively loaded in (A) and (B) were mixed and resolved by 2D-gel electrophoresis followed by autoradiography. The gel was exposed immediately after the experiment in such a way that both [^{3}H]leucine- and ^{32}P-labeled proteins could be detected. Only a portion of each autoradiograph showing the 28K protein is shown with the acidic end at left. Arrows indicate the position of the a, b and c isoforms.

revealed the induction of a number of proteins with respective M_r of 85 000, 62 000, 50 000 and 28 000. The 28K protein synthesis was markedly stimulated after 1 h heat-shock and reached a plateau at 4 h. The electrophoretic mobility of the [^{3}H]leucine-labeled 28K protein was found identical to that of [^{32}P]labeled 28K protein observed upon TPA stimulation (not shown). In order to ascertain that the 28K phosphoprotein and the 28K protein synthetized under stress conditions is the same protein, we performed in parallel 2D-gel electrophoresis fractionation of [^{3}H]leucine- and ^{32}P-labeled proteins from respectively heat-shock- and phorbol ester-treated cells. As shown in Fig. 5A, the 28K protein synthetized upon heat-shock was resolved as two main [^{3}H]leucine-labeled isoforms, named 'a' and 'b', with respective pI of 6.5 and 5.9, while a 3rd isoform named 'c' with pI 5.7 appeared slightly labeled. None of these 28K isoforms were observed on 2D-gel from control cells (not shown). The 28K protein phosphorylated upon TPA treatment was observed, as already shown in Fig. 2, as 2 ^{32}P-labeled isoforms 'b' and 'c' with respective pI of 5.9 and 5.7 (Fig. 5B). As a consequence, isoform 'a' very likely represented the unphosphorylated 28K protein as previously described in other cell lines [28,29]. According to co-migration on the same gel of [^{3}H]leucine- and ^{32}P-labeled extracts, the 'b' and 'c' isoforms synthetized upon heat-shock were identical to those phosphorylated in response to TPA (Fig. 5C). Further autoradiography of the co-migration gel after ^{32}P was allowed to decay for 60 days, led to the same pattern as the one observed in Fig. 5A (not shown). Taken together, our results strongly suggest that the 28K PKC substrate described in mesangial cells probably belongs to the stress protein family. In any event, activation of PKC in mesangial cells can be monitored by studying the phosphorylation of 2 major prominent protein kinase C substrates: an 80K acidic phosphoprotein which could be very close to the MARCKS protein, and a 28K protein which could be a stress protein.

Activation of PKC by phorbol esters leads to a marked reduction in the affinity of the EGF receptor for EGF [30], a process called transmodulation. Figure 6A demonstrates that in mesangial cells, TPA and phorbol 12,13-dibutyrate (PDBu), at 100 ng/ml, decreased the

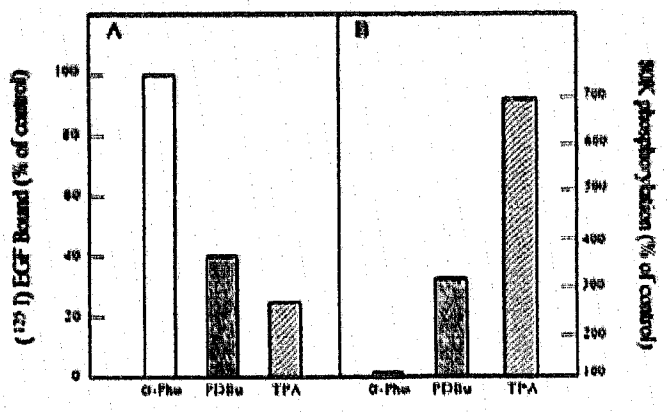

Fig. 6. TPA and PDBu, but not 4α-phorbol, induce [^{125}I]EGF transmodulation and 80K phosphorylation in mesangial cells. (A) Cells were incubated for 1 h at 37°C in binding medium containing 100 ng/ml TPA (hatched bar), 100 ng/ml PDBu (dotted bar) or 100 ng/ml 4α-phorbol (α-Pho, open bar). At the end of incubation, the binding of [^{125}I]EGF to intact cells was determined at 4°C as described in section 2. Values are the mean of 3 independent experiments. Results are expressed as the percentage of the mean of the control values (14 pg/10^6 cells). (B) Cells were labeled with [^{32}P]phosphoric acid for 2 h and then treated with 100 ng/ml TPA (hatched bar), 100 ng/ml PDBu (dotted bar) or 100 ng/ml 4α-phorbol (α-Pho, open bar) for 15 min. Triton extracts were analysed as in Fig. 3.

binding of [^{125}I]EGF after 1 h exposure. PDBu was less potent than TPA corresponding to the lesser efficiency of this compound for activating PKC [14]. Similar to its lack of effect on mitogenesis (Table I), 4α-phorbol was completely unable to induce EGF transmodulation. Fig. 6B shows the same order of potency of TPA, PDBu and 4α-phorbol (no effect) for inducing the 80K protein phosphorylation when compared to that for inhibiting EGF binding (Fig. 6A).

In conclusion, we have demonstrated that the phorbol ester TPA can induce DNA synthesis in mesangial cells suggesting a positive role for PKC in mesangial cell proliferation. Moreover, we have shown that activation of PKC leads to the phosphorylation of 2 major 28K and 80K proteins. Studies of the phosphorylation of these 2 PKC substrates could be a useful means for investigating the mechanism of action of various peptides which are mitogenic for mesangial cells.

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