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Phorbol 12-myristate 13-acetic acid inhibits PTP1B activity in human mesangial cells
A possible mechanism of enhanced tyrosine phosphorylation

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Abstract Activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetic acid (PMA) stimulates DNA synthesis in human glomerular mesangial cells. Incubation of these cells with PMA stimulates the tyrosine phosphorylation of a set of proteins ranging from 110 to 39 kDa with different time kinetics. PMA inhibits total protein tyrosine phosphatase (PTPase) activity in these cells. Immunoprecipitation of PTP1B, an intracytoplasmic tyrosine phosphatase, with subsequent assay of the immunobeads for PTPase shows a significant inhibition of its activity in PMA-treated cells. Immunoblot analysis of mesangial cell lysates using the same antibody revealed that PMA does not affect the level of this 50 kDa PTP1B protein. These data indicate that inhibition of total PTPase, and specifically PTP1B, activity may provide a mechanism for stimulation of tyrosine phosphorylation by PMA in these cells and thereby contribute to its mitogenic effect.

Key words: Phorbol ester; Tyrosine phosphorylation; Protein tyrosine phosphatase; PTP1B

1. Introduction

Increased DNA synthesis and proliferation of mesangial cells is a common abnormality and may be a marker of activity in glomerular diseases [1,2]. PKC may mediate the mitogenic effect of cytokines expressed in renal disease. We have recently shown that PKC may mediate PDGF-induced DNA synthesis in these cells [3]. The potent PKC activator, PMA, has been widely used to study the role of PKC in different biological processes [4]. We have shown that PMA stimulates DNA synthesis in mesangial cells [5]. PMA has also been shown to stimulate tyrosine phosphorylation of several proteins in cultured cells [6–9]. The specific tyrosine kinase(s) involved in this phosphorylation process are not known. However, the level of tyrosine phosphorylation depends upon the competing action of kinases and phosphatases which catalyze phosphorylation and dephosphorylation reactions, respectively. Therefore it is important to identify and characterize the protein tyrosine phosphatases (PTPases) involved in this dynamic equilibrium.

PTPases are a family of enzymes which include receptor type and intracellular proteins [10]. The non-receptor PTPases comprise a catalytic domain fused to regulatory motifs which have homology with cytoskeletal proteins, SH2 domains and PEST sequences [10]. The most characterized non-transmembrane PTPase is PTP1B, which was originally isolated from human placenta and found to be expressed ubiquitously [11,12]. It has recently been shown that PTP1B is phosphorylated by PKC [13]. In this study we demonstrate that PMA inhibits total PTPase and PTP1B activity in human mesangial cells. Inhibition of PTPases by PMA may contribute to stimulation of tyrosine phosphorylation and mitogenesis in response to PMA.

2. Experimental

2.1. Materials

PMA, myelin basic protein (MBP), Triton X-100, phenylmethylsulfonyl fluoride (PMSF) and charcoal (Norit A) were obtained from Sigma, St. Louis, MO. Aprotinin was purchased from Miles Laboratory, Kankakee, IL. $p60^{c\text{-}src}$ tyrosine kinase was from Oncogene Science, Uniondale, NY. Monoclonal antiphosphotyrosine antibody and polyclonal PTP1B antibody were obtained from UBI, Lake Placid, NY. A431 epidermal carcinoma cell lysate was purchased from Transduction Laboratories, Lexington, KY. ECL immunoblotting reagents were purchased from Amersham, Arlington Heights, IL. Protein A-Sepharose beads were obtained from Pharmacia, Piscataway, NJ. All other chemicals were of analytical grade.

2.2. Cell culture

Mesangial cells from collagenase-treated glomeruli of normal human kidney were cultured in Waymouth's medium in the presence of 17% fetal bovine serum as described before [3]. Confluent cells were made quiescent by incubation in serum-free medium for 3 days. Quiescent cells were treated with 100 nM PMA for indicated periods of time.

2.3. Immunoblot analysis

The cells were lysed in buffer A (50 mM HEPES, pH 7.4, 5 mM EDTA, 0.25 M sucrose, 1% Triton X-100, 115 μM PMSF, 0.05% aprotinin, 5 mM benzamidine) for 30 min at 4°C. After centrifugation at 10,000 × g for 30 min the protein concentration in the supernatant was determined. Equal amounts of cell lysate were analyzed on 7.5% polyacrylamide gels, and separated proteins were electrophoretically transferred to polyvinyl membranes. Western immunoblotting analysis of the proteins was performed using either a monoclonal antiphosphotyrosine antibody or a polyclonal PTP1B antibody. The detailed procedure has been published recently [14].

2.4. Immunoprecipitation

Equal amounts of cleared cell lysate were incubated with 3 μl (1 μg) of PTP1B antibody on ice for 30 min. 15 μl of protein A-Sepharose beads (50% v/v slurry) were added and incubated at 4°C on a rocking platform for 2 h. The beads were washed once with buffer A without aprotinin, twice with double-distilled water, and once with 25 mM HEPES, pH 7.3, and then assayed for PTPase activity.

2.5. PTPase assay

MBP substrate was tyrosine phosphorylated by $p60^{c\text{-}src}$ and [γ-^{32}P]ATP as previously described [15]. The PTPase assay was performed according to the method of Tonks et al. [11,12] with minor modification. Briefly, 0.5 μg cell lysate or the PTP1B immunoprecipitates were incubated with tyrosine-phosphorylated ^{32}P-labeled MBP. The reaction was carried out in 50 μl buffer (25 mM HEPES, pH 7.3, 5 mM EDTA, 10 mM DTT) at 30°C for 30 min. Incubation was

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stopped by adding 0.75 ml of acidic charcoal mixture. The mixture was centrifuged at 10.000 × *g* for 10 min and the $^{32}P_i$ released in the 400 μl supernatant was determined in a scintilation counter. Initially the assay was standardized to show linearity with protein ranging from 0.125 to 5.0 μg, and the dephosphorylation was linear within 1.0 μg of protein. The PTPase activity was expressed relative to the control, set as 100%. Independent experiments were used to express the data as mean ± S.E.M.

3. Results

3.1. Stimulation of tyrosine phosphorylation in mesangial cells

Addition of 100 nM PMA to human mesangial cells stimulated tyrosine phosphorylation of multiple proteins in a time-dependent manner (Fig. 1). Tyrosine phosphorylation of a 110 kDa protein was stimulated within 5 min of PMA addition and persisted for 20 min. The tyrosine-phosphorylated proteins migrating at 85, 79 and 44 kDa were stimulated at 10 min after the addition of PMA. Tyrosine phosphorylated 60 and 39 kDa proteins were stimulated at 20 and 30 min, respectively. The identity of all these proteins, with the exception of the 44 kDa one, is not known. The 44 kDa tyrosine-phosphorylated protein is most likely mitogen-activated protein kinase. Nearly similar patterns of tyrosine phosphorylation were reported in rat mesangial cells [9]. These data indicate that PMA stimulates tyrosine phosphorylation of several proteins with different time kinetics.

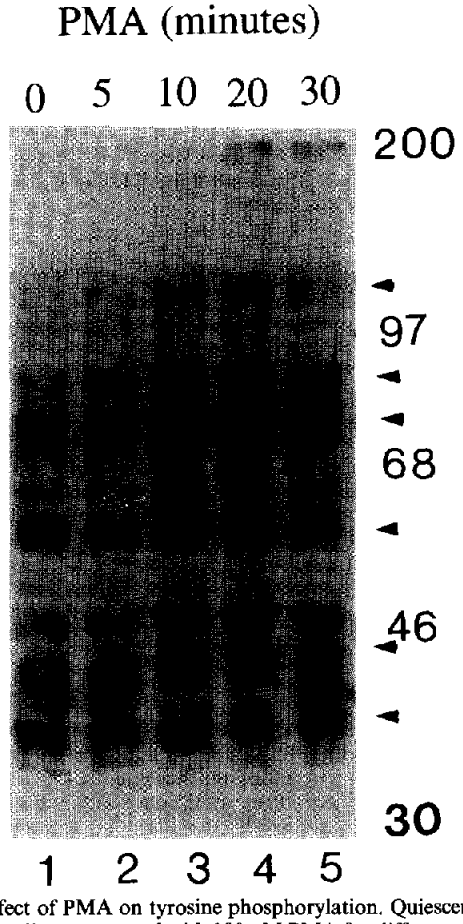

Fig. 1. Effect of PMA on tyrosine phosphorylation. Quiescent human mesangial cells were treated with 100 nM PMA for different periods of time. 50 μg of cell lysate was analyzed by immunoblotting using anti-phosphotyrosine antibody as described in section 2. Molecular weight markers are shown in kDa. The arrowheads indicate the protein bands the tyrosine phosphorylation of which is enhanced by PMA. Their molecular weights from top to bottom are 110, 85, 79, 60, 44 and 39 kDa.

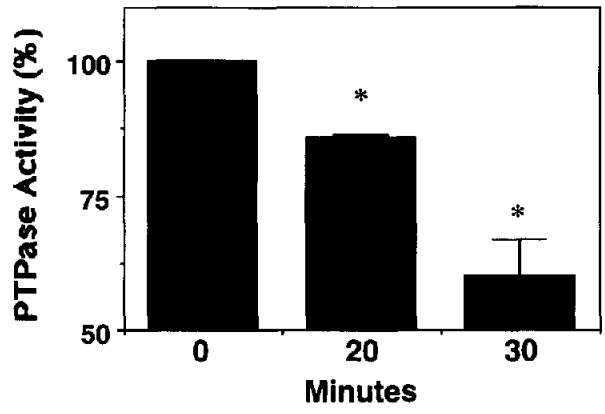

Fig. 2. Effect of PMA on total PTPase activity. Quiescent human mesangial cells were stimulated with 100 nM PMA for the indicated periods of time. PTPase activity was determined in 0.5 μg of cell lysate as described in section 2. $^*P < 0.05$.

3.2. Inhibition of PTPase activity

Since the level of tyrosine-phosphorylated proteins in the cell depends upon the balanced activity of tyrosine kinases and tyrosine phosphatases, we measured total PTPase activity of mesangial cell lysate after stimulation with PMA. At 20 min the activity was reduced to 85%, whereas at 30 min a significant reduction to 60% of the basal activity was observed (Fig. 2). These results indicate that the PMA-induced stimulation of tyrosine phosphorylation seen above (Fig. 1) may be partially mediated by the inhibition of PTPases.

3.3. Effect of PMA on PTP1B activity

The intracellular PTPase, PTP1B, is expressed ubiquitously. We first characterized a polyclonal antibody raised against an N-terminal fusion protein of PTP1B in an immune complex phosphatase assay. Different amounts of mesangial cell lysate were immunoprecipitated with PTP1B antibody. The immunobeads were assayed for PTPase activity. The data show that PTP1B antibody does not inhibit the phosphatase activity of the immunoprecipitated enzyme (Fig. 3A). To test the effect of PMA on the activity of PTP1B, mesangial cells were stimulated with PMA for 20 or 30 min. After protein determination, equal amounts of cell lysate were immunoprecipitated with PTP1B antibody. The immunobeads were assayed for PTPase activity. The data show that PMA inhibited PTP1B activity by 25 and 43% at 20 and 30 min, respectively (Fig. 3B). To test the effect of PMA on the level of PTP1B protein, we determined the amount of the enzyme by immunoblot analysis (Fig. 4). In mesangial cells, the antibody identified the 50 kDa PTP1B protein (indicated by an arrow). PMA treatment did not affect the level of PTP1B protein (Fig. 4, compare lanes 2 and 3 with 1). These data suggest that the inhibitory effect of PMA on PTP1B activity was not due to the loss of PTP1B protein. Furthermore PTP1B antibody recognized a 46 kDa protein in mesangial cells (Fig. 4, lanes 1–3). This protein is not

present in the human epidermal carcinoma cell line, A431 (Fig. 4, lane 4).

4. Discussion

Cytokines and other agonists act on receptors with intrinsic tyrosine kinase activity, as well as on non-tyrosine kinase receptors, to stimulate PKC. Thus activation of PKC in vivo is a central signal that mediates the action of several agonists. We show that PMA, a potent activator of PKC [4], stimulates tyrosine phosphorylation of different proteins with specific time kinetics (Fig. 1), as has been reported in rat mesangial cells [9].

To investigate the role of PTPases, we measured total PTPase activity of cell lysates stimulated with PMA (Fig. 2). Inhibition of PTPase activity is consistent with the observation that PMA stimulates tyrosine phosphorylation of a panel of proteins. Although we cannot rule out the possibility of activation of tyrosine kinase(s) by PMA, at least its inhibitory effect on PTPase activity may provide an alternative mechanism of stimulation

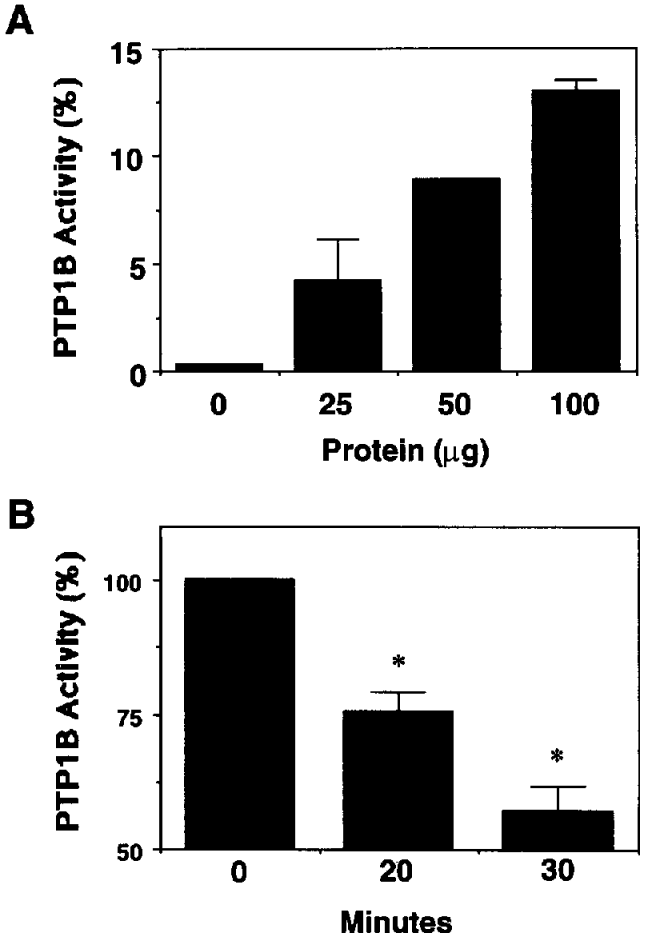

Fig. 3. (A) Detection of PTP1B activity in human mesangial cells. Different amounts of cell extracts were immunoprecipitated with PTP1B antibody, as described in section 2, and the immunobeads were assayed for PTPase activity. (B) Effect of PMA on PTP1B activity. Cells were stimulated as in Fig. 2. Immunoprecipitates of PTP1B from equal amounts of cell lysate were assayed for PTPase activity, as described in section 2. $*P < 0.01$.

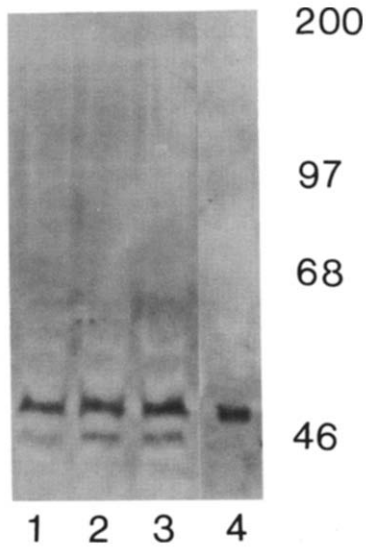

Fig. 4. Immunoblot analysis of PTP1B protein. Quiescent human mesangial cells were stimulated with PMA for different periods of time. Cells were lysed in buffer A and 50 μg of the cell lysate (lanes 1–3) and 10 μg of A431 cell lysate (lane 4) were separated on an SDS gel and analyzed by immunoblotting using PTP1B antibody. Lane 1, unstimulated; lanes 2 and 3, 20 and 30 min after PMA treatment. Molecular weight markers are shown in kDa. The arrow indicates the 50 kDa PTP1B enzyme in both cells.

of tyrosine phosphorylation in mesangial cells. Our results are in contrast to a recent report that PMA decreases the EGF-dependent tyrosine phosphorylation of proteins in NIH 3T3 fibroblasts transfected with EGF receptor via stimulation of PTPases [16]. Taken together these data suggest that the effect of PMA is likely to be cell-type specific.

Similar to several other signalling enzymes, phosphorylation of PTPases may provide a mechanism of their enzymatic activation. Thus phosphorylation of a 55 kDa membrane-bound PTPase by protein kinase A or PKC stimulates its enzymatic activity [17]. The best-characterized intracytoplasmic PTPase, PTP1B, is a phosphoprotein, and the phosphorylation pattern is cell cycle specific [18]. It has recently been shown that PMA treatment of HeLa cells stimulates phosphorylation of Ser^{378} of PTP1B [13]. This same residue is also phosphorylated in vitro by purified PKC [13]. In this report, we provide evidence that treatment of human mesangial cells with PMA inhibits PTP1B activity (Fig. 3B). Whether this inhibition is due to the phosphorylation of PTP1B by PKC in vivo or involves modulation of PTP1B by some other protein(s) which is regulated by PKC, is not clear. However, immunoblot analysis of mesangial cell lysate revealed that the PTP1B protein is not regulated by PMA treatment (Fig. 4). This result indicates that the inhibition of PTP1B activity is not due to down-modulation of the protein by PMA. Along with 50 kDa PTP1B, we also detected a 46 kDa protein in mesangial cells (Fig. 4). This protein was not found in A431 epidermal carcinoma cells or in other cell types studied previously [18]. Taken together these data provide the first evidence that in mesangial cells PMA inhibits PTP1B activity. Inhibition of PTP1B may mediate, at least partially, the stimulation of tyrosine phosphorylation, and thereby contribute to its mitogenic effect.

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