

# PKC $\alpha$ regulates thrombin-induced PDGF-B chain gene expression in mesangial cells

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**Abstract** Thrombin is a potent mitogen for mesangial cells and stimulates PDGF B-chain gene expression in these cells. It also activates phospholipase C (PLC) resulting in an increase in cytosolic  $\text{Ca}^{2+}$  and diacylglycerol (DAG) that are the physiological activators of protein kinase C (PKC). Immunoprecipitation of specific PKC isotypes from thrombin-stimulated mesangial cells with subsequent measurement of their enzymatic activity shows activation of  $\text{Ca}^{2+}$ -dependent PKC  $\alpha$  and  $\text{Ca}^{2+}$ -independent PKC  $\zeta$  in a time dependent manner. Optimum activation of both of these isozymes was obtained at 60 minutes. PKC  $\alpha$  activity increased 83% over basal while activity of PKC  $\zeta$  increased 104%. Prolonged exposure of mesangial cells to phorbol myristate acetate (PMA) inhibited the enzymatic activity of PKC  $\alpha$  but not PKC  $\zeta$ . This inhibition of PKC  $\alpha$  had no effect on thrombin-induced DNA synthesis but abolished PDGF B-chain gene expression induced by thrombin. These data provide the first evidence that PKC  $\alpha$  activation is necessary for thrombin-induced PDGF B-chain gene expression but not for thrombin-induced DNA synthesis.

**Key words:** Thrombin; PKC isotype; Mitogenic signaling

## 1. Introduction

Activation of the coagulation pathway with thrombin production and fibrin deposition are features of severe vascular and proliferative glomerular diseases [1,2]. Through its proteolytic activity, thrombin cleaves a tethered ligand and leads to activation of the receptor which is a member of the seven transmembrane spanning class of receptors linked to G-proteins [3]. Thrombin stimulates production of diacylglycerol (DAG) and inositol 1,4,5-tris-phosphate ( $\text{IP}_3$ ) resulting from the hydrolysis of phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) [4,5].  $\text{IP}_3$  stimulates intracellular mobilization of  $\text{Ca}^{2+}$ . These second messengers including DAG and  $\text{Ca}^{2+}$ , are known to activate protein kinase C (PKC) [6]. Multiple isozymes of PKC have been isolated by molecular cloning [6,7]. They fall into three groups. Group A consists of four classical PKCs ( $\alpha$ PKC),  $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$  and they are  $\text{Ca}^{2+}$ -dependent for their enzymatic activity. Group B comprises four novel PKCs ( $\text{nPKC}$ )  $\delta$ ,  $\epsilon$ ,  $\theta$  and  $\eta$ . Group C consists of two atypical PKC isotypes ( $\text{aPKC}$ ),  $\zeta$  and  $\lambda$  [6,7]. PKC isozymes in group B and C are

$\text{Ca}^{2+}$ -independent and indeed they lack the  $\text{Ca}^{2+}$ -binding C2 domain in their protein sequence [6,7]. Recently it has been shown that in chinese hamster embryo fibroblasts, thrombin promotes translocation of PKC  $\alpha$  and  $\epsilon$  to the membrane from cytosol [8]. This observation suggests that thrombin causes activation of these PKC isotypes since translocation of PKC seems to be associated with activation of its enzymatic activity.

We have previously reported that thrombin is a potent mitogen for mesangial cells and that it stimulates phosphoinositide (PI) turnover and mobilization of intracellular  $\text{Ca}^{2+}$  in these cells [9]. Using immunoblot analysis, Saxena et al. reported the presence of PKC  $\alpha$ , PKC  $\beta$ I and  $\gamma$  in rat mesangial cells [10]. In a separate study, Huwiler et al. showed that rat mesangial cells express only PKC  $\alpha$  among the  $\text{Ca}^{2+}$ -dependent isozymes. These authors could not detect PKC  $\beta$  or  $\gamma$  [11]. Using highly specific cDNA probes representing each  $\text{Ca}^{2+}$ -dependent PKC isotypes and immunoprecipitation using isotype-specific antibodies followed by measurement of enzymatic activity, we identified PKC  $\alpha$  as the predominant  $\text{Ca}^{2+}$ -dependent PKC isozyme in human mesangial cells. This PKC isotype was activated by PDGF [12]. Other groups have recently reported the presence of  $\text{Ca}^{2+}$ -independent PKC  $\delta$ ,  $\epsilon$  and  $\zeta$  isotypes in mesangial cells [11,13]. In this report, we demonstrate that thrombin stimulates PKC  $\alpha$  and PKC  $\zeta$  in human mesangial cells. We also provide the first evidence that inhibition of PKC  $\alpha$  but not PKC  $\zeta$  is associated with abolition of PDGF B-chain mRNA expression without any effect on thrombin-induced mitogenesis.

## 2. Experimental

### 2.1. Materials

Purified human  $\alpha$ -thrombin and PMA were purchased from Sigma, St. Louis, MO. PKC assay kit and PKC isozyme specific antibodies were obtained from Gibco-BRL, Grand Island, N.Y. Protein A sepharose CL 4B was from Pharmacia, Piscataway, NJ.  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (3000 Ci/mmol) was obtained from Dupont-NEN, Boston, MA. Western blotting kit (ECL Western blotting protocols) was purchased from Amersham. All other chemicals were analytical grade.

### 2.2. Cell culture

Normal portions of human nephrectomy samples or human kidney judged unsuitable for transplantation were used to prepare glomeruli for cell culture. Glomeruli were isolated from kidney cortex by sieving technique [14] and mesangial cells were cultured and characterized as we described previously [15]. The cells were grown in Waymouth's medium in the presence of 17% fetal calf serum [12] and were used between passage 6 and 11. The cells were made quiescent by incubation in serum free Waymouth's media for 48 h. Cells were incubated with  $6 \times 10^{-7}$  M PMA for 48 h to downregulate PKC.  $10^{-7}$  M PMA was added to study its effect on PDGF B-chain gene expression.

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### 2.3. Immunocomplex PKC assay

This assay has been utilized for measuring activities of serine threonine kinases and tyrosine kinases [16,17]. The principle of the assay is to use specific antibodies, which do not inhibit the activity of the enzyme, to immunoprecipitate the enzyme protein and use the bound protein on the immunebeads to assay the activity of the enzyme. We used isotype specific polyclonal antibodies raised against peptide sequences present in the V3 region of PKC  $\alpha$  (ala-gly-asn-lys-val-ile-ser-pro-ser-glu-asg-arg-arg-gln), PKC  $\beta$  (gly-pro-lys-thr-glu-glu-lys-thr-ala-asn-thr-ile-ser-lys-phe-asg) and PKC  $\gamma$  (asn-tyr-pro-leu-glu-leu-tyr-glu-arg-val-arg-thr-gly). For  $\text{Ca}^{2+}$ -independent PKC  $\epsilon$ ,  $\delta$  and  $\zeta$ , C-terminal peptides were used to raise the antibody. For PKC  $\delta$ , a dodecapeptide (ser-phe-val-asn-pro-lys-tyr-glu-gln-phe-leu-glu) was used. For  $\epsilon$  also, a 12-mer peptide (lys-gly-phe-ser-tyr-phe-gly-glu-asg-leu-met-pro) was used. For PKC  $\zeta$  a 16-mer peptide (gly-phe-glu-tyr-ile-asn-pro-leu-leu-leu-ser-ala-glu-ser-val) was used for immunization. Quiescent mesangial cells were stimulated with 5 units/ml thrombin for the time periods indicated. For PKC downregulation experiments, the cells were pretreated with  $6 \times 10^{-7}$  M PMA for 48 h before the addition of thrombin. The cells were washed with ice cold PBS and lysed in homogenization buffer (20 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25  $\mu\text{g}/\text{ml}$  aprotinin, 25  $\mu\text{g}/\text{ml}$  leupeptin) at  $4^\circ\text{C}$  for 30 min. The cell lysate was centrifuged at  $10,000 \times g$  for 30 min at  $4^\circ\text{C}$ . Protein concentration in the supernatant was determined using Bio-Rad protein assay reagent. Equal amounts of cell lysates were immunoprecipitated with different PKC isotype-specific antibodies as described [12]. The PKC activity in the immunoprecipitates was measured using reagents purchased as a kit from Gibco-BRL. The details of the assay have recently been described by us [12]. Myelin basic protein (MBP)-derived peptide was used as a substrate for assaying PKC  $\alpha$  and a Ser<sup>25</sup>-substituted peptide obtained from pseudosubstrate region of PKC  $\alpha$  was used for PKC  $\epsilon$ ,  $\delta$  and  $\zeta$  [18]. It has recently been shown that this is a better substrate for  $\text{Ca}^{2+}$ -independent PKC isotypes than MBP-derived substrate [18].

### 2.4. Immunoblot analysis

Immunoblotting was performed as we described recently [19].

### 2.5. Measurement of DNA synthesis

DNA synthesis was measured as [ $^3\text{H}$ ]thymidine incorporation into trichloroacetic acid insoluble material as previously described [12].

### 2.6. RNase protection assay

RNase protection assay was performed using radiolabeled RNA transcript of PDGF B chain gene encompassing exon 6 and 7. The detailed method is published elsewhere [20].

### 2.7. Data analysis

The significance of the data was determined by Student's *t*-test.

## 3. Results

### 3.1. Activation of PKC $\alpha$ and PKC $\zeta$ in mesangial cells

Immunoprecipitation of PKC  $\alpha$  from control and thrombin-stimulated mesangial cells followed by *in vitro* immune complex assay of PKC showed activation of this isozyme (Fig. 1A). Thrombin stimulated PKC  $\alpha$  activity within 5 min (150%). Peak activity of 83% over basal was obtained after 60 min of stimulation. Similar immunocomplex kinase assays were performed with  $\text{Ca}^{2+}$ -independent PKC  $\delta$ ,  $\epsilon$  and  $\zeta$ . The data showed significant stimulation of PKC  $\zeta$  activity (21% of basal) within 10 min (Fig. 1B). The optimum activation (104% of basal) was obtained at 60 min. No activation of PKC  $\delta$  and  $\epsilon$  were obtained in response to thrombin. These data demonstrate activation of both  $\text{Ca}^{2+}$ -dependent PKC  $\alpha$  and  $\text{Ca}^{2+}$ -independent PKC  $\zeta$  by thrombin in human mesangial cells. To demonstrate the specificities of the antibodies used in these immunoprecipitation experiments, we performed immunoblot experiments of human mesangial cell lysates in the presence and absence of immuniz-

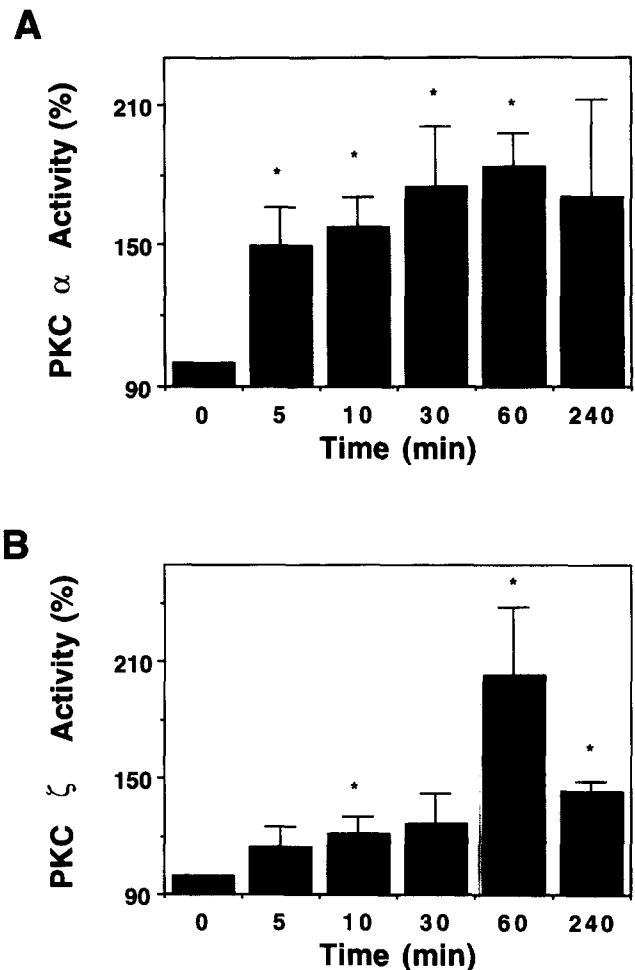


Fig. 1. Activation of PKC  $\alpha$  and PKC  $\zeta$  in human mesangial cells. Quiescent cells were stimulated with 5 units/ml thrombin for different periods of time. 100  $\mu\text{g}$  of cell lysate was immunoprecipitated with PKC  $\alpha$  specific and PKC  $\zeta$  specific antibodies, respectively. The immunocomplex PKC activity was measured using isotype specific substrate peptides as described in section 2. Mean with S.E.M. of three independent experiments each done in duplicate. '\*' indicates that the increase is significant as compared to controls. Panel A, PKC  $\alpha$ ; \* $P < 0.02$ . Panel B, PKC  $\zeta$ ; \* $P < 0.05$ .

ing PKC isotype-specific peptides. As positive control, we used rat brain extract which is rich in different  $\text{Ca}^{2+}$ -dependent and independent PKC isotypes. The data show that the PKC  $\alpha$  specific antibody recognized an 80 kDa protein, which is competed by the immunizing peptide (Fig. 2, PKC  $\alpha$  panel, compare lane 1 with lane 3). The data also demonstrate the specificity of PKC  $\zeta$  antibody which recognizes a 71 kDa protein being competed by the  $\zeta$  specific immunizing peptide (Fig. 2, PKC  $\zeta$  panel, compare lane 1 with lane 3). Specificity of PKC  $\delta$  and PKC  $\epsilon$  antibodies was also tested in a similar manner (data not shown).

### 3.2. Activation of PKC $\alpha$ is not required for thrombin-induced DNA synthesis

To examine the role of PKC  $\alpha$  in thrombin-induced mitogenesis, the enzyme was downregulated by pretreatment of human mesangial cells with PMA. Thrombin-induced mito-

genesis was measured as the amount of [ $^3\text{H}$ ]thymidine incorporation into DNA (Fig. 3A). The data show a 12-fold increase in DNA synthesis by thrombin in these cells. Downregulation of PKC in the presence of  $6 \times 10^{-7}$  M PMA for 48 h did not have any significant effect on thrombin-induced DNA synthesis (Fig. 3A). To verify the downregulating effect of PMA on PKC isotypes, we measured the activity of PKC  $\alpha$  and PKC  $\zeta$  after PMA pretreatment. The data show a loss of more than 90% of PKC  $\alpha$  activity whereas PMA pretreatment of mesangial cells had no effect on PKC  $\zeta$  activity (Fig. 3B and C). This observation confirms the previously published finding that phorbol ester does not deplete PKC  $\zeta$  in mesangial cells [21].

### 3.3. Involvement of PKC $\alpha$ in thrombin-induced PDGF B chain mRNA expression

PDGF B-chain gene expression is a delayed early response in mesangial cells. We have previously demonstrated that in addition to its mitogenic activity, thrombin stimulates PDGF B-chain mRNA expression [9]. To investigate the role of PKC in this process, PKC  $\alpha$  was downregulated by prolonged preincubation of these cells with PMA. The cells were then stimulated with thrombin and the expression of PDGF B-chain mRNA was examined by RNase protection assay. The data show that PMA and thrombin stimulate PDGF B-chain gene expression (Fig. 4). Downregulation of PKC  $\alpha$  by pretreatment of mesangial cells with PMA abolished the expression of PDGF B-chain mRNA induced by thrombin. These results indicate that thrombin-induced PDGF B-chain gene expression is sensitive to PKC  $\alpha$  downregulation. In contrast PKC  $\zeta$  does not seem to play a role in this process since the activity of PKC  $\zeta$  is not inhibited by pretreatment of mesangial cells with PMA (Fig. 3C).

## 4. Discussion

In mesangial cells, the protease growth factor thrombin stimulates PDGF gene expression and mitogenesis [9]. The thrombin signal transduction pathways leading to different biological effects are not precisely identified. Recent observations indicate that activation of PKC by growth factor and cytokines plays

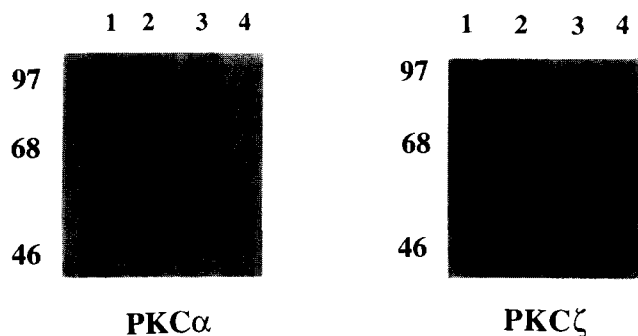


Fig. 2. Specificity of PKC  $\alpha$  and PKC  $\zeta$  antibodies. 10  $\mu\text{g}$  of human mesangial cell lysate (lanes 1 and 3) and 10  $\mu\text{g}$  of rat brain extract (lanes 2 and 4) were separated on SDS polyacrylamide gel, transferred to PVDF membrane and immunoblotted with anti-PKC  $\alpha$  and PKC  $\zeta$  antibody in the presence and absence of the immunizing peptides as described in section 2. Lanes 1 and 2 are in the absence and lanes 3 and 4 are in the presence of the competing peptide. Molecular weight markers in kDa are shown in margins.

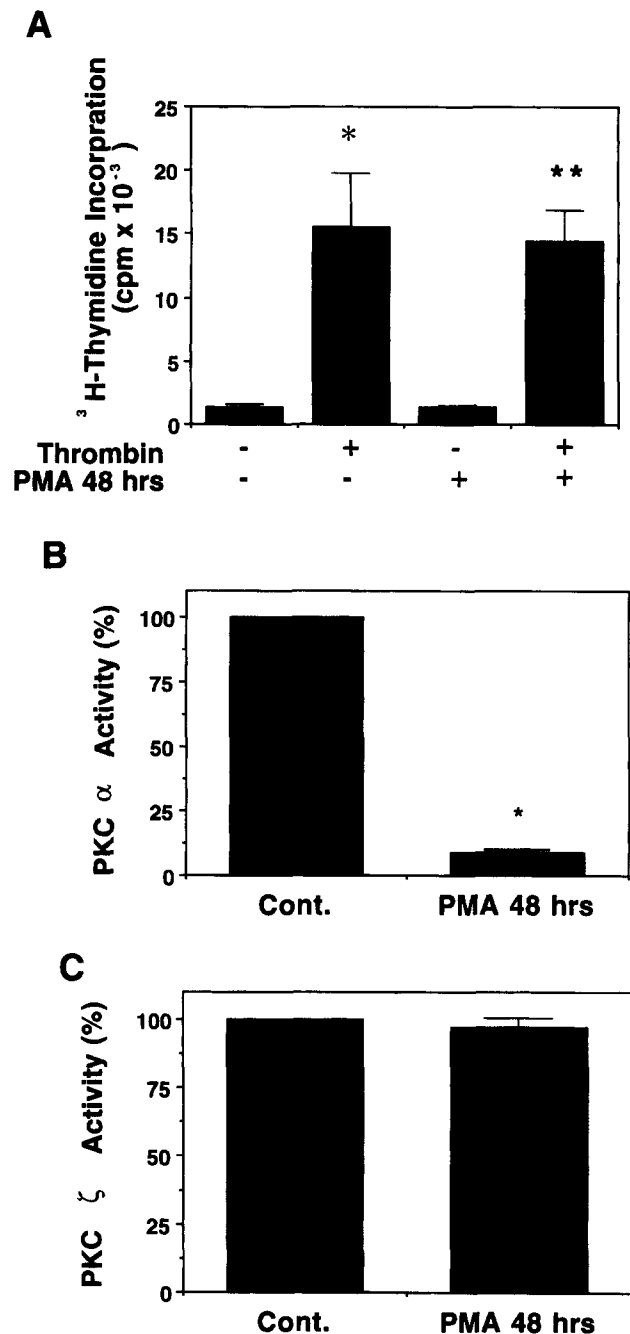


Fig. 3. (Panel A) Effect of PKC downregulation on thrombin-induced DNA synthesis. Quiescent human mesangial cells were pretreated with  $6 \times 10^{-7}$  M PMA for 48 h before addition of thrombin or PMA. DNA synthesis was measured as amount of [ $^3\text{H}$ ]thymidine incorporation into TCA-insoluble precipitate as described in section 2. Results of three independent experiments each done in replicates of six wells. \* $P < 0.001$  vs. unstimulated. \*\* $P < 0.001$  vs. PMA stimulation. (Panels B and C). Effect of PMA on PKC  $\alpha$  and PKC  $\zeta$  activity. Human mesangial cells were treated with  $6 \times 10^{-7}$  M PMA for 48 h and the basal PKC  $\alpha$  and PKC  $\zeta$  activity was measured after immunoprecipitation of the enzyme from 100  $\mu\text{g}$  of cell lysate with isoform specific antibodies as described in section 2. Cont, control. Mean of three independent experiments each done in quadruplicate. \* $P < 0.05$ .

a major role in exerting their physiological effects [6,22]. In mesangial cells both  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent

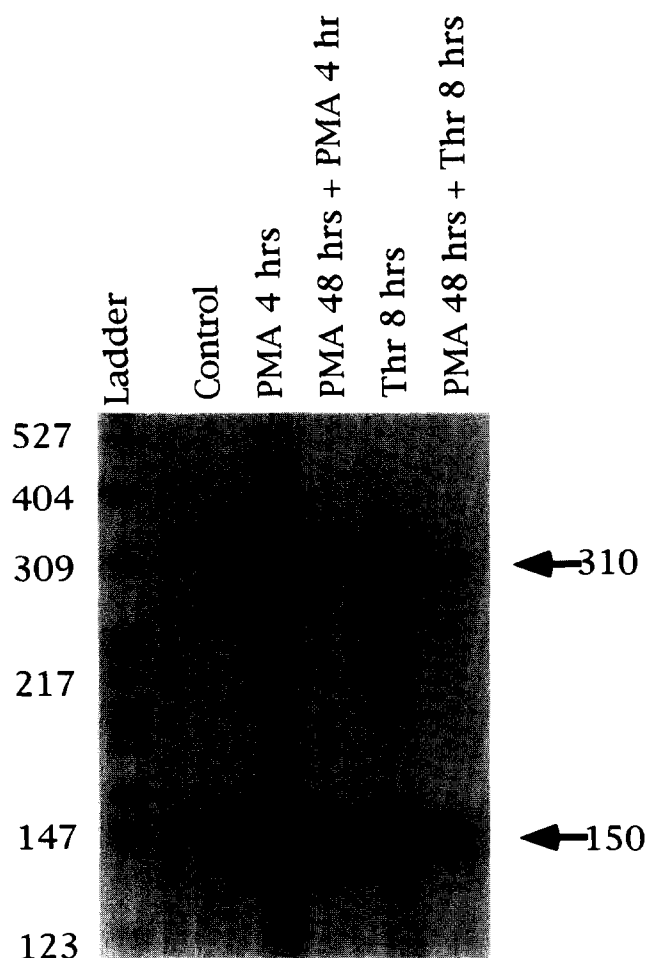


Fig. 4. Effect of PKC  $\alpha$  downregulation on thrombin-induced PDGF B-chain mRNA expression in human mesangial cells. The cells were treated with  $6 \times 10^{-7}$  M PMA for 48 hours. Total RNA was isolated from PMA (4 hours) and thrombin (8 hours) stimulated cells and analyzed by RNase protection assay for PDGF B-chain message as described in section 2. MspI digest of pBR322 DNA was labeled and used as a size marker as shown on the left margin. Two protected fragments of 310 and 150 bp represent the PDGF B-chain mRNA species.

isotypes of PKCs are expressed [10–13,21]. The specific biological role of these PKC isotypes are not known. In this report we provide evidence that in mesangial cells, thrombin stimulates the  $\text{Ca}^{2+}$ -dependent PKC  $\alpha$  and  $\text{Ca}^{2+}$ -independent PKC  $\zeta$ . Upregulation of PKC  $\alpha$  has been shown to be required for cell growth [22]. We demonstrate that inhibition of PKC  $\alpha$  activity by prolonged incubation of cells with PMA which results in downregulation of this enzyme had no effect on thrombin-induced DNA synthesis in mesangial cells (Fig. 3A,B). This indicates that thrombin utilizes PKC  $\alpha$ -independent signal transduction pathways for mitogenesis. Indeed we have recently shown that tyrosine phosphorylation is required for thrombin-induced DNA synthesis in mesangial cells [23]. We have previously shown that thrombin stimulates PI turnover to synthesize DAG and  $\text{IP}_3$  [9]. The latter stimulates mobilization of  $\text{Ca}^{2+}$  from intracellular stores. Production of these second messengers is a prerequisite for activation of PKC  $\alpha$ . However activation of PKC  $\zeta$  is independent of the presence of DAG and

$\text{Ca}^{2+}$  [18,24]. Downregulation of PKC by prolonged incubation of cells with PMA did not inhibit PKC  $\zeta$  activity in mesangial cells (Fig. 3C). Whether activation of PKC  $\zeta$  is required for thrombin-induced DNA synthesis is not yet clear.

PDGF B-chain gene induction is a known biological response of mesangial cells to certain mitogens including thrombin [9]. The mechanism by which thrombin exerts this effect is not known. Previous studies showed that activation of PKC by phorbol ester results in the induction of gene expression [25]. In the present study we demonstrate that PMA stimulates PDGF B-chain gene expression and this induction is dependent on the activation of PKC  $\alpha$  (Fig. 4). In addition, downregulation of PKC  $\alpha$  by prolonged incubation with PMA abolished thrombin-induced PDGF B-chain gene expression indicating the requirement of PKC  $\alpha$  for this effect of thrombin (Fig. 4). Since PKC  $\zeta$  activity is unchanged under these conditions (Fig. 3C), we conclude that thrombin-mediated activation of this isozyme of PKC is not sufficient for induction of PDGF-B-chain mRNA expression. These data demonstrate that activation of different PKC isotypes even in the same cell regulates specific biological response to agonist. Taken together, these data indicate that PKC  $\alpha$  and PKC  $\zeta$  are the downstream target molecules for thrombin-mediated signal transduction pathways in mesangial cells and that PKC  $\alpha$  regulates the expression of PDGF B-chain gene. Further studies will be necessary to explore a direct link between mitogenesis and activation of PKC  $\zeta$  in mesangial cells.

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