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SUPPRESSIVE EFFECT OF TRANSFORMING GROWTH FACTOR-β ON THE PHOSPHORYLATION OF ENDOGENOUS SUBSTRATES BY CONVENTIONAL AND NOVEL PROTEIN KINASE C IN PRIMARY CULTURED MOUSE EPIDERMAL CELLS

Kiyotaka Nishikawa, Satoshi Yamamoto, Haruna Nagumo, Chino Otsuka and Ryuichi Kato

Department of Pharmacology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan

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SUMMARY: The effect of transforming growth factor-β (TGF-β) on the endogenous protein phosphorylation caused by phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC), was examined in primary cultured mouse epidermal cells. PMA markedly stimulates phosphorylation of endogenous proteins, i.e. KP-1 and KP-2, through Ca^{2+}-dependent conventional PKC (cPKC), and KP-10 through Ca^{2+}-independent novel PKC (nPKC) in intact epidermal cells. TGF-β strongly suppressed the PMA-stimulated phosphorylation of these three proteins. Rate of dephosphorylation of these phosphorylated proteins was not affected by TGF-β. Treatment of epidermal cells with TGF-β decreased cPKC activity both in cytosolic and particulate fractions, but not nPKC activity. These results indicate that TGF-β suppresses cPKC- and nPKC-mediated endogenous protein phosphorylation in intact epidermal cells, but the mechanisms of suppression are different.

Transforming growth factor-β (TGF-β) is a family of polypeptides that modulate growth and differentiation of a wide variety of cells (1, 2). In human and mouse keratinocytes, TGF-β is known to be a potent and reversible growth inhibitor (3). Recent studies have pointed out the variation in the TGF-β-induced signalling pathways, i.e. involvement of protein kinases (4-7), protein phosphatases (8), and guanine nucleotide binding proteins (9-11). It has been reported that TGF-β type II and V receptors are functional transmembrane serine/threonine protein kinases (12, 13). However, the precise mechanism by which TGF-β exerts its effect is not fully understood.

Protein kinase C (PKC) is a family of enzymes which are classified into two major groups, Ca^{2+}-dependent conventional PKC (cPKC) and Ca^{2+}-independent novel PKC (nPKC) and is known to play crucial roles in the signal transduction of a variety of extracellular stimuli, such as hormones and growth factors (14). It has been reported that TGF-β induces apparent redistribution

ABBREVIATIONS:

TGF-β, transforming growth factor-β; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MEM, minimum essential medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PS, phosphatidylserine.

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of PKC activity from cytosol to membrane (15, 16). Guerrin *et al.* reported that TGF-β interferes with PKC pathway without inhibiting 28,000 Mr endogenous protein phosphorylation stimulated by phorbol 12-myristate 13-acetate (PMA), a potent PKC activator, in breast adenocarcinoma subline (17). However, the effect of TGF-β on the cellular signalling pathways mediated by different types of PKC, i.e. cPKC and nPKC, has not been elucidated.

We recently have found endogenous substrate proteins for cPKC, i.e. KP-1 (pI 4.7 / 23,000 Mr) and KP-2 (pI 4.7 / 20,700 Mr), and for nPKC, i.e. KP-10 (pI 4.7 / 25,500 Mr), in primary cultured mouse epidermal cells (18). Phosphorylation levels of these proteins are markedly enhanced by PMA treatment in intact cells. In the present study, we demonstrate the suppressive effect of TGF-β on the PMA-stimulated phosphorylation of these endogenous proteins.

MATERIALS AND METHODS

Primary culture of mouse epidermal cells
Primary cultured mouse epidermal cells were prepared as described previously (19). The epidermal cells were cultured in Ca^{2+}-free minimum essential medium (MEM) supplemented with 10% Chelex-treated (Ca^{2+}-deprived) fetal calf serum (FCS) and 50 μM $CaCl_2$ (final concentration). After 24 hr, the medium was switched to Ca^{2+}-free MEM supplemented with 0.5% Ca^{2+}-deprived FCS and 50 μM $CaCl_2$. The cells were cultured further for 24 hr.

Protein phosphorylation in intact epidermal cells
Cultured epidermal cells (35-mm dish) were treated with or without 3 ng/ml TGF-β (R & D systems Inc., Minneapolis, MN) for the indicated time periods, and washed twice with phosphate-deprived Locke's solution (buffer A). The cells were then radiolabeled with 7.4 MBq/ml [^{32}P]H_3PO_4 (ICN Biomedicals Inc., Irvine, CA) for 30 min at 37 °C. Thereafter the cells were washed and treated with 30 nM PMA (Sigma Chemical Co., St Louis, MO) for 10 min in the presence or absence of 3 ng/ml TGF-β. At the end of incubation, the cells were lysed with 0.25 ml sodium dodecyl sulfate (SDS)-buffer (62.5 mM Tris, 2% SDS, 5% mercaptoethanol and 5% glycerol, pH 6.8).

Dephosphorylation of endogenous phosphoproteins
Cultured epidermal cells (35-mm dish) were washed twice with buffer A and incubated in 1 ml of new buffer A containing 7.4 MBq/ml [^{32}P]H_3PO_4 for 30 min at 37 °C. Thereafter the cells were washed and treated with 30 nM PMA for 10 min. After washing, the cells were further incubated in buffer A in the presence or absence of 3 ng/ml TGF-β for the indicated time periods. After incubation the cells were lysed with the SDS buffer.

Polyacrylamide gel electrophoresis
Two hundred microliter aliquots of cell lysates were analyzed by two-dimensional polyacrylamide gel electrophoresis (PAGE), and the radioactivities of phosphoproteins were measured as described previously (18).

Preparation of PKC from epidermal cells
Epidermal cells (100-mm dish) were incubated in the presence or absence of 3 ng/ml TGF-β for 1 hr. After incubation, the cytosol and particulate fractions of epidermal cells were separated (18). The preparations of cPKC and nPKC in cytosol and particulate fractions were obtained using Mono Q (HR 5/5) column as described previously (18).

Protein kinase assay
Protein kinase activity was measured as described previously (18). Briefly, endogenous substrate fraction containing KP-1, -2 and -10 was prepared from epidermal cells using Mono Q and Superose 12 columns (Pharmacia, Piscataway, NJ). The reaction mixture consists of 20 mM Tris-HCl (pH 7.5), 0.01 % Triton X-100, 20 mM 2-mercaptoethanol, 1 mM PMSF, 1.12 mM EGTA, 1.12 mM EDTA, 6.12 mM $MgCl_2$, 10 μM (1 μCi) [γ-^{32}P]ATP (ICN Biomedicals Inc., Irvine,

CA), 40 μg/ml phosphatidylserine (PS), 100 μl of enzyme fraction, and 20 μl of substrate fraction at a total volume of 250 μl in the presence or absence of 2.12 mM $CaCl_2$ or 4 μM PMA. After incubation at 30 °C for 3 min, the reaction was stopped by the addition of 25 μl of ice-cold 100 % trichloroacetic acid, followed by the addition of 20 μg of bovine serum albumin as a carrier. Precipitates were analyzed by SDS-PAGE, and the radioactivity of each protein was measured and shown as arbitrary unit as described previously. cPKC activity was determined by subtracting the radioactivity obtained in the absence of co-factors from that in the presence of Ca^{2+} and PS. nPKC activity was determined by subtracting the radioactivity obtained in the absence of co-factors from that in the presence of PS and PMA.

RESULTS AND DISCUSSION

We have previously shown that the phosphorylation of endogenous proteins, designated as KP-1 (pI 4.7 / 23,000 Mr), KP-2 (pI 4.7 / 20,700 Mr) and KP-10 (pI 4.7 / 25,500 Mr), are markedly stimulated by PMA in primary cultured mouse epidermal cells (18). As shown in Fig. 1-A, PMA-stimulated phosphorylation of these three proteins was markedly suppressed by simultaneous addition of TGF-β. Pretreatment of the cells with TGF-β for 0.5 to 2 hours also suppressed the PMA-induced phosphorylation of these three proteins (Fig. 1-B). Partial suppression of KP-2 phosphorylation by TGF-β may reflect the fact that KP-2 is constitutively phosphorylated to a certain extent even in unstimulated cells (Fig. 1-A and Ref. 18). It has been reported that TGF-β activates protein serine/threonine phosphatase in human keratinocytes (8). To examine this possibility, the effect of TGF-β on the dephosphorylation of KP-1, -2 and -10 were examined. As clearly shown in Fig. 2, the rate of dephosphorylation of KP-1, -2 and -10 was not affected by TGF-β, indicating that the suppression by TGF-β of the PMA-stimulated protein phosphorylation is not due to enhanced dephosphorylation.

Although, in the absence of PMA, TGF-β alone did not affect the phosphorylation of KP-1, -2 and -10, phosphorylation of another protein (pI 4.8 / 17,900 Mr) was enhanced by TGF-β (indicated by open arrowhead in Fig. 1-A). This enhancement, however, was not always reproducible. Recent studies have shown that TGF-β induces rapid phosphorylation of cyclic AMP responsive element binding protein (CREB) (5) and heat shock protein 28 (7), although the precise mechanism is not known. Moreover, TGF-β type II and V receptors are functional transmembrane serine/threonine kinases (12, 13). Therefore, TGF-β-induced phosphorylation of the above endogenous protein in epidermal cells may reflect such phosphorylation stimulating actions of TGF-β.

As reported previously, KP-1 and -2 are direct substrates for Ca^{2+}- and phospholipid-dependent protein kinase (cPKC) and KP-10 is a substrate for Ca^{2+}-independent, phospholipid-dependent protein kinase (nPKC) in intact mouse epidermal cells (18). Effects of TGF-β on the activities of these two types of PKC in epidermal cells were examined next. After treatment of the cells with 3 ng/ml TGF-β for 1 h, cPKC and nPKC activities both in cytosolic and particulate fractions were determined. As shown in Fig. 3, the cPKC activity, which phosphorylates KP-1 and -2, was decreased both in cytosolic and particulate fractions by treating the cells with TGF-β.

It has been reported that TGF-β induces translocation of PKC in cultured astrocytes (16) and in microvessels isolated from rat brain (15). Enhanced translocation of PKC, however, cannot explain the suppressive effect of TGF-β on PMA-stimulated protein phosphorylation, since

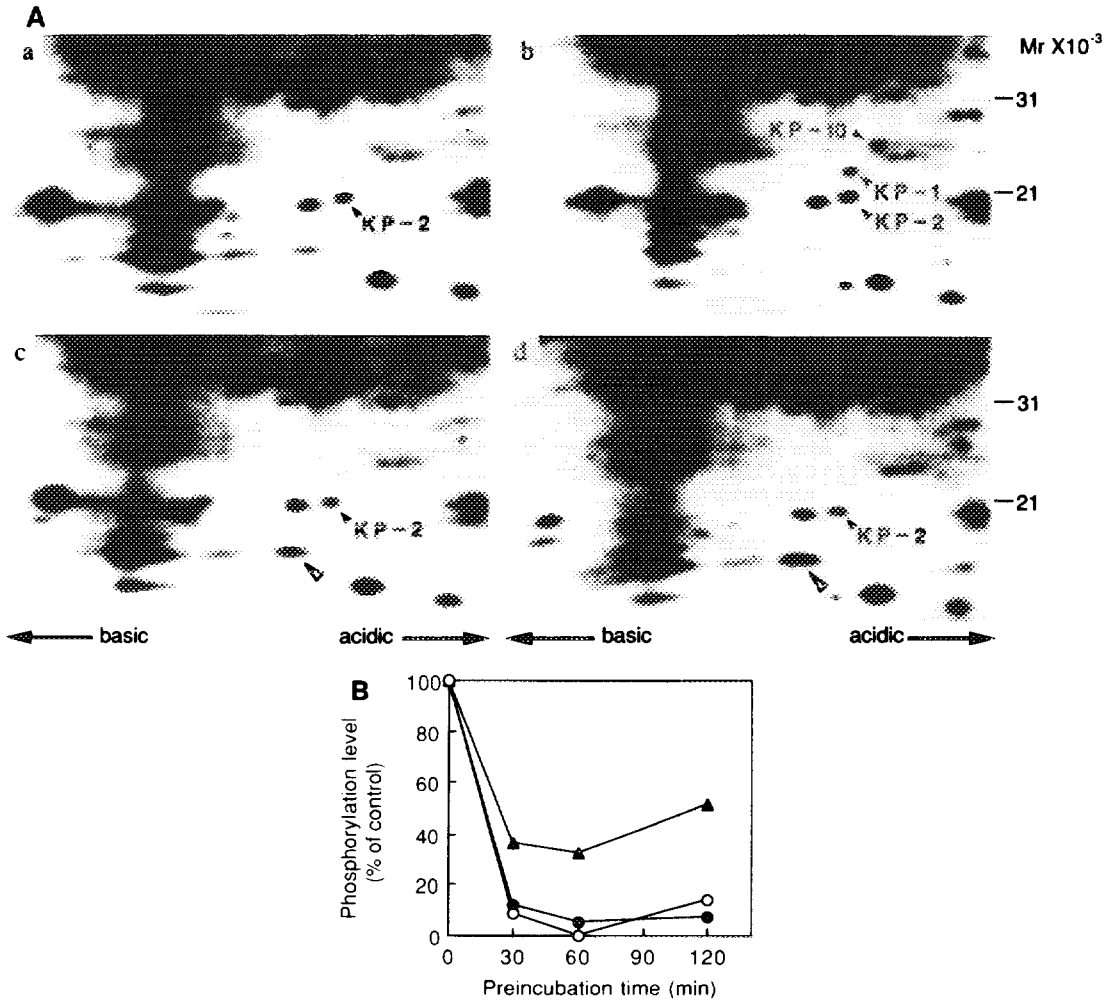

Fig. 1. Effects of TGF-β on the PMA-stimulated phosphorylation of endogenous proteins in epidermal cells. (A) Cultured epidermal cells were prelabelled with $[^{32}P]H_3PO_4$ for 30 min, then the cells were treated with 30 nM PMA (b), 3 ng/ml TGF-β (c), or PMA plus TGF-β (d) for 10 min. (a) shows non-treated cells. Thereafter the cells were lysed and subjected to two-dimensional PAGE. (B) Cultured epidermal cells were treated with 3 ng/ml TGF-β for the indicated time periods and washed with buffer A. Thereafter the cells were labelled with $[^{32}P]H_3PO_4$ for 30 min, then the cells were treated with 30 nM PMA for 10 min. Thereafter the cells were lysed and subjected to two-dimensional PAGE. The radioactivities of KP-1, -2 and -10 were measured. The phosphorylation level of each protein without TGF-β pretreatment was set at 100 %. Closed circle, closed triangle and open circle indicate KP1, -2 and -10, respectively.

enhancement of translocation of PKC would augment the effect of PMA. Moreover, the suppressive effect of TGF-β was observed even after pretreating the cells with TGF-β for 30 min, which induces maximal translocation of PKC (15, 16). It may be possible that the suppression of cPKC activity by TGF-β results from the down-regulation of cPKC which follows after membrane translocation of this enzyme. This possibility, however, seems not to be the case, because TGF-β suppressed PMA-stimulated phosphorylation within 10 min (Fig. 1-A) and the down-regulation of PKC is not observed within 10 min after the addition of TGF-β (15, 16). Although at present the

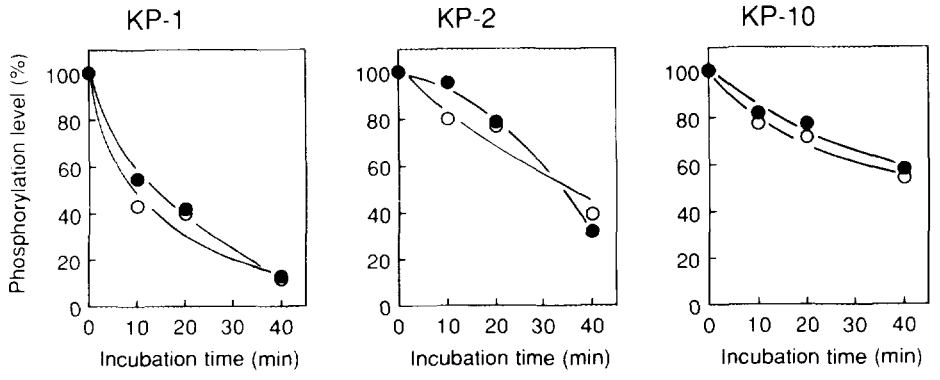

Fig. 2. Effect of TGF-β on the dephosphorylation of endogenous phosphoproteins. Cultured epidermal cells were prelabelled with [^{32}P]H_3PO_4 for 30 min, then the cells were treated with 30 nM PMA for 10 min. Thereafter the cells were washed to remove PMA and further incubated in buffer A in the presence (open circle) or absence (closed circle) of 3 ng/ml TGF-β for the indicated time periods. The phosphorylation level of each protein after PMA stimulation was set at 100 %.

cellular mechanism by which TGF-β decreased cPKC activity is unknown, it may be possible that TGF-β activates another pathway which enhances cPKC degradation, such as activation of proteases responsible for its degradation. Anyhow, it is possible that the suppression of PMA-stimulated phosphorylation of KP-1 and -2 by TGF-β is due to the decrease in cPKC activity.

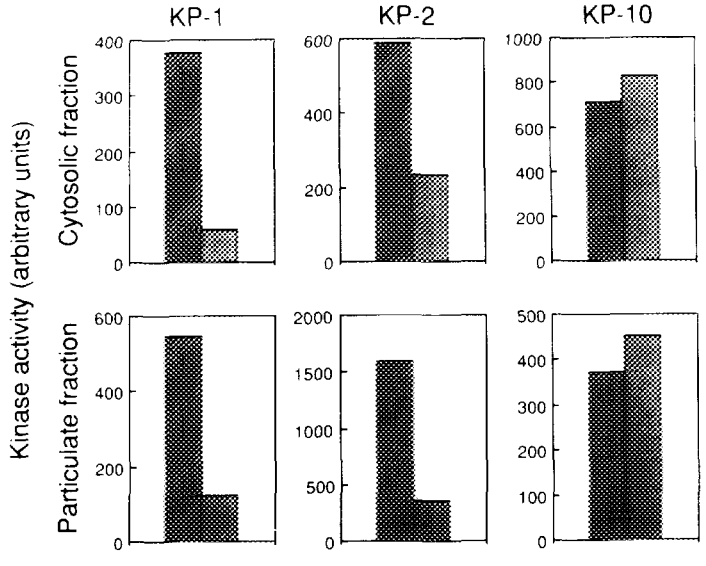

Fig. 3. Effects of TGF-β treatment on PKC activities in epidermal cells. Cultured epidermal cells were incubated in the absence (□) or presence (■) of 3 ng/ml TGF-β for 1 hr. Then the cytosol and particulate fractions were separated. The preparations of cPKC and nPKC in cytosol and particulate fractions were obtained using Mono Q (HR 5/5) column. cPKC activity, which phosphorylates KP-1 and -2, was determined in the presence of Ca^{2+} and PS. nPKC activity, which phosphorylates KP-10, was determined in the presence of PS and PMA. The values shown are representative of two different experiments.

Contrary to the case of cPKC, the nPKC activity, which phosphorylates KP-10, both in cytosolic and particulate fractions was not decreased by treating the cells with TGF-β. Therefore the suppression of PMA-stimulated KP-10 phosphorylation by TGF-β may not be explained by the decrease in nPKC activity. At present the precise mechanism of the suppression of KP-10 phosphorylation by TGF-β in intact cells is unknown. Our present results indicate that TGF-β suppresses cPKC- and nPKC-mediated endogenous protein phosphorylation in intact epidermal cells but the mechanisms of suppression are different .

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