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Inhibition of epidermal growth factor-dependent protein tyrosine phosphorylation by phorbol myristate acetate is mediated by protein tyrosine phosphatase activity

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Abstract

Incubation of HER14 cells with phorbol myristate acetate (PMA) decreases epidermal growth factor (EGF)-dependent protein tyrosine phosphorylation, except for a 40-kDa MAP kinase II-like protein, whose tyrosine phosphorylation is further enhanced. The inhibitory effect of PMA on EGF-dependent protein tyrosine phosphorylation is reversed if cells are pre-incubated with a combination of Na_3VO_4 and NaF, two known inhibitors of protein tyrosine phosphatase activity. Protein tyrosine phosphatase activity of cell homogenate was measured on immunopurified EGF receptor, and was found to be enhanced in PMA-treated cells. These data suggest that the inhibitory effect of PMA on EGF-dependent protein tyrosine phosphorylation in HER14 cells may be mediated by protein tyrosine phosphatase activity.

Key words: Epidermal growth factor; Protein tyrosine phosphorylation; Protein tyrosine phosphatase; Phorbol myristate acetate; Protein kinase C; Fibroblast

1. Introduction

EGF-receptor (EGF-R)[1] is a transmembrane protein tyrosine kinase, which upon ligand binding, undergoes an autophosphorylation on tyrosine residues and phosphorylates other protein substrates [1]. Tumor promoter phorbol esters decrease high affinity binding of EGF, and inhibit the tyrosine kinase activity of EGF-R [2–5]. This regulation is supposedly due to a protein kinase C (PKC)-mediated serine/threonine phosphorylation of EGF-R [6–8].

In addition, it was proposed that phosphorylation of EGF-R at Thr^{654} by PKC is necessary for inhibition of EGF-R kinase activity, as well as for negative control of EGF-induced mitogenesis and receptor internalization [9–11]. Phosphorylation of EGF-R at threonine 669 [12,13] might also regulate EGF-R signaling. Recently, Rosner and co-workers [14] suggested that pp42 MAP kinase decreases tyrosine phosphorylation of EGF-R by activating a vanadate-sensitive protein tyrosine phosphatase. Although several studies have implicated protein tyrosine phosphatases [15] in the regulation of peptide growth factor-mediated protein tyrosine phosphorylation [16–19], the role of these enzymes in the PKC-mediated inhibition of EGF-R signaling was never investigated. In this study, we show that protein tyrosine phosphatase activity in HER14 cells may mediate the inhibitory effect of PMA on EGF-dependent protein tyrosine phosphorylation.

2. Experimental

NIH-3T3 cells transfected with the human EGF receptor (HER14 cells) and the human epidermoid carcinoma cell line A431 (kindly provided by Dr. J. Schlessinger) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum for A431 cells, or 10% calf serum for HER14 cells, 2 mM glutamine and streptomycin (50 mg/ml)/penicillin (50 IU/ml), at 5% CO_2 in air and 37°C. Before starting the experiment, cells (50% to 90% confluence) were starved 4 h in fresh media supplemented with 20 mM HEPES (pH 7.4), and 0.5% serum. After cell treatment with PMA (Sigma) or with EGF (Toyobo Co), cell media was aspirated, followed by scraping of cells off the plates in buffer A (20 mM HEPES,150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin and 1 μg/ml leupeptin, pH 7.4.) with or without Na_3VO_4 (100 μM) and NaF (20 mM). Lysate was left at ice cold temperature for 30 min, then was clarified by centrifugation for 3 min at 16,000 × *g* in a microcentrifuge. In the resulting supernatant (cell homogenate) proteins were measured by the Bio-Rad method [20] and matched, before being subjected to SDS-PAGE, followed by immunoblotting with anti-phosphotyrosine antibody as described [21].

^{32}P-labeled EGF-R for the protein tyrosine phosphatase activity assay was prepared as follows: Cell homogenate was prepared from confluent A431 cells as described for HER14 cells, and was incubated for 90 min at 4°C under agitation with EGF-R monoclonal antibody,

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Abbreviations: dimethylsulfoxide, DMSO; Dulbecco's modified Eagle's medium, DMEM; epidermal growth factor, EGF; ethylene glycol bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, EGTA; phenylmethylsulfonyl fluoride, PMSF; phorbol myristate acetate, PMA; protein kinase C, PKC.

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mAb108 [22] (kindly provided by Dr. J. Schlessinger) that was coupled to Protein A-Sepharose. The immunoprecipitate was washed twice with buffer B (20 mM HEPES, 350 mM NaCl, 10% Glycerol, 0.1% Triton X-100, pH 7.4) and twice with buffer B′ (Buffer B with only 150 mM NaCl). The immunoprecipitate was then resuspended in 250 μl of 20 mM HEPES (pH 7.4) in the presence of $MgCl_2$ (10 mM), 5–10 μCi [^{32}P]ATP (6,000 Ci/mmol, from NEN), and cold ATP (100 μM). Phosphorylation was carried out at room temperature and was terminated 30 min later by centrifugation. The beads were washed three times with buffer B′, then were resuspended in 300–400 μl of 20 mM HEPES (pH 7.4) before utilization. Protein tyrosine phosphatase activity was measured by incubating 40 μl of HER14 cell homogenate (54 μg) with 20 μl of ^{32}P-labeled EGF-R as a substrate. 20 min later, reaction was terminated by adding 60 μl of 2× Laemmli sample buffer. Samples were boiled four min, and centrifuged in a microcentrifuge. An aliquot of the supernatant was subjected to SDS-PAGE (7.5%). The gel was dried in a Bio-Rad gel drier and than exposed to Kodak film for 0.5 to 3 h at −70°C. Protein tyrosine phosphatase activity in each sample was estimated as a decrease of radioactivity associated with EGF receptor band compared to buffer A-treated substrate. The band corresponding to EGF-R was cut and counted in a beta counter by the Cerenkof method. The assay was performed in the linear range of ^{32}P-labeled EGF-R dephosphorylation with respect to time of incubation and protein amount.

High affinity binding assay of EGF was performed as described [11]. Cells were seeded in 24-well plastic plates (5×10^4 cells/well). 48 h later, cells ($20–25 \times 10^4$/well) were rinsed with binding buffer (DMEM supplemented with 20 mM HEPES and 1 mg/ml BSA) and incubated at 37°C with the drugs to be studied. Cells were then transferred on ice, and 10 min later, ^{125}I-EGF (100 μCi/μg, from ICN) (0.5 nM final) was added to the cells for an additional 3 h. Wells where non specific binding was to be determined were treated with cold EGF (50 nM final). Reaction was terminated by aspirating the media, and rinsing the cells three times with binding buffer. Then cells were solubilzed in 0.8 ml NaOH (0.5 N) at 37°C for one hour, then radioactivity was counted in an LKB gamma counter.

3. Results

Addition of 50 nM EGF to HER14 cells induced protein tyrosine phosphorylation of several bands including that of the EGF-R (170 kDa). This effect was inhibited when cells were preincubated with 1 μM PMA, with the exception of a 40 kDa band whose tyrosine phosphorylation was enhanced (Fig. 1, line 3). The 40 kDa band has been identified as a MAP kinase (Errasfa and Stern, personal communication).

When cells were pre-treated with a combination of Na_3VO_4 (0.1 mM) and NaF (20 mM), an enhancement of tyrosine phosphorylation was observed on several proteins (Fig. 1, line 4). After 50 nM EGF treatment in the presence of Na_3VO_4 and NaF, protein tyrosine phosphorylation also occurred on the EGF-R band (170 kDa) and was increased on the 40 kDa band (Fig. 1, lines 5). Na_3VO_4 and NaF prevented the inhibitory effect of PMA on EGF-dependent protein tyrosine phosphorylation, while tyrosine phosphorylation of the 40 kDa band was further enhanced (Fig. 1, line 6). When cells were pretreated with EGTA, the effect of PMA on EGF-dependent protein tyrosine phosphorylation was not significantly modified (Fig. 1, line 9), indicating that Ca^{2+} may not be required.

Since the inhibitory effect of PMA on EGF-dependent protein tyrosine phosphorylation was reversed by a combination of two protein tyrosine phosphatase inhibitors, we investigated the effect of PMA on protein tyrosine phosphatase activity in HER14 cells. Protein tyrosine phosphatase activity was measured in cell homogenate in the presence of ^{32}P-labeled immunopurified EGF-R as a substrate in vitro (Fig. 2). Cell homogenate exhibited a PTPase activity toward ^{32}P-labeled EGF-R. This activity was found to be significantly enhanced in PMA-treated cells in a concentration- and time-dependent manner (Fig. 2,A,B). About 35% of EGF-R was dephosphorylated by cell homogenate derived from control cells, while 59% of this substrate was dephosphorylated by cell homogenate of PMA-treated cells (Fig. 2, see inset on page 16 of this manuscript).

PMA is known to decrease high-affinity binding of EGF [10]. We confirmed this finding and also investigated the effect of Na_3VO_4 and NaF on high-affinity binding of EGF. As shown in Table 1, PMA decreased high-affinity binding of ^{125}I-EGF to HER14 cells. The combination of NaF and Na_3VO_4, also decreased the high-affinity binding of ^{125}I-EGF, which did not change in the presence of PMA (Table 1). This result appears paradoxical because EGF-dependent protein tyrosine

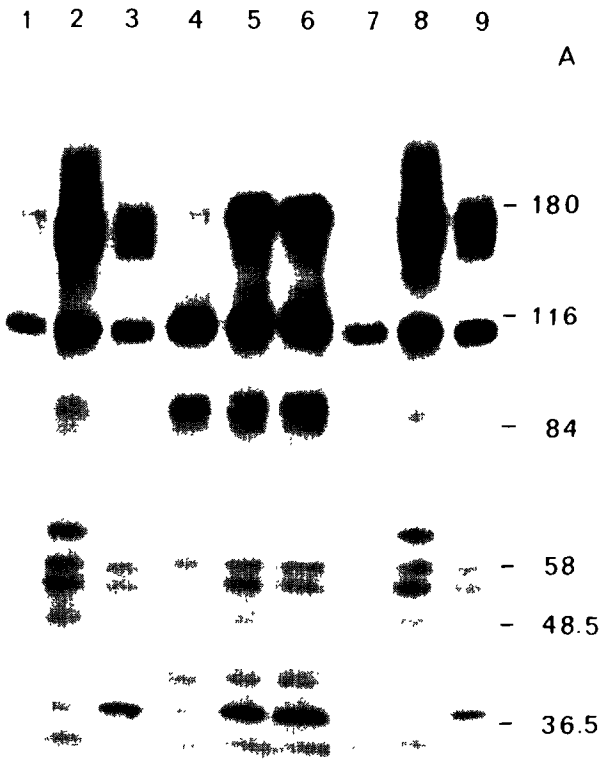

Fig. 1. Inhibition of in vivo EGF-dependent protein tyrosine phosphorylation by PMA is reversed by Na_3VO_4 and NaF, but not EGTA. Serum-starved HER14 cells were incubated 30–40 min with a combination (lines 4–6) of 100 μM Na_3VO_4 and 20 mM NaF, or with 2.5 mM ethylene glycol bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) (lines 7–9). Then dimethylsulfoxide (DMSO, 0.2%) (lines 1,2,4,5,7,8) or 1 μM PMA (lines 3,6 and 9) was added for five min. 50 nM EGF was added for an additional five min (lines 2,3,5,6,8,9). Tyrosine phosphorylated proteins were analysed as described in Section 2.

phosphorylation is enhanced by treatment of cells with Na_3VO_4 and NaF in the presence of PMA (Fig. 1).

4. Discussion

We have found that the major effect of PMA on regulation of EGF-dependent protein tyrosine phosphorylation in HER14 cells was an inhibition of protein tyrosine phosphorylation, accompanied by the enhancement of tyrosine phosphorylation of a 40 kDa MAP kinase-related protein. To investigate the role of protein tyrosine phosphatases in the EGF-dependent inhibition of protein tyrosine phosphorylation by PMA, we used two inhibitors of these enzymes, Na_3VO_4 and NaF. Elevation of the basal tyrosine phosphorylation of several proteins and the increase in EGF-dependent tyrosine phosphorylation of the 40 kDa protein in the presence of the inhibitors is consistent with inhibition of protein tyrosine phosphatases. Consequently, the reversal of the inhibitory effect of PMA on EGF-dependent protein tyrosine phosphorylation in HER14 cells by the protein tyrosine phosphatase inhibitors suggests a mediating role for protein tyrosine phosphatase activity in this action of PMA. This is supported by the observation that protein tyrosine phosphatase activity in cell homogenate was significantly enhanced in PMA-treated cells.

The involvement of protein tyrosine phosphatases in the PMA effect on EGF-dependent protein tyrosine phosphorylation is compatible with a study by Brautigan and Pinault [15] which showed a PMA-dependent stimulation of protein tyrosine phosphatase activity on an artificial substrate. A recent finding by Rosner and coworkers [19] showed that pp42 MAP kinase enhances EGF-R dephosphorylation through the stimulation of a vanadate-sensitive protein tyrosine phosphatase. This mode of regulation of EGF-R is similar to our present finding, in that the negative regulation of EGF-R signaling induced by a serine/threonine MAP kinase is mediated by a protein tyrosine phosphatase [19]. As MAP kinase is known to be activated by peptide growth factors and phorbol esters [23,24], the question remains as to whether or not MAP kinase mediates the PMA effect on protein tyrosine phosphatase activation.

In conclusion, EGF-dependent protein tyrosine phosphorylation in HER14 cells may be inhibited by PMA through a PKC-activated protein tyrosine phosphatase and the decrease of high-affinity binding of EGF caused by PMA may not be relevant to the inhibition of EGF-dependent protein tyrosine phosphorylation.

Table 1
Effect of PMA, Na_3VO_4 and NaF on high-affinity binding of EGF in HER14 cells

HER14 cells	Exp 1	Exp 2	Exp 3
Control	3,327	4,916	3,527
PMA	629	1,229	739
Na_3VO_4/NaF	523	624	1,358
Na_3VO_4/NaF/ PMA	518	619	858

Cells were seeded in 24-well plastic plates (5×10^4 cells/well). 48 h later, cells ($20–25 \times 10^4$/well) were rinsed with binding buffer (DMEM supplemented with 20 mM HEPES and 1 mg/ml BSA) and incubated for 40 min at 37°C in the same buffer containing Na_3VO_4 (100 μM) and NaF (20 mM). Then PMA (1 μM) or DMSO (0.2%) was added for an additional 15 min. Plates were transferred on ice and 10 min later, ^{125}I-EGF (0.5 nM final) was added to the cells for an additional 3 h. Wells where non specific binding was to be determined were also treated with cold EGF (50 nM final). Reaction was terminated by aspirating the media, and rinsing the cells three times with binding buffer. Then cells were solubilized in 0.8 ml NaOH (0.5 N) for one hour at 37°C, and radioactivity was counted in an LKB gamma counter. Data in cpm are from three representative experiments. nd, not determined.

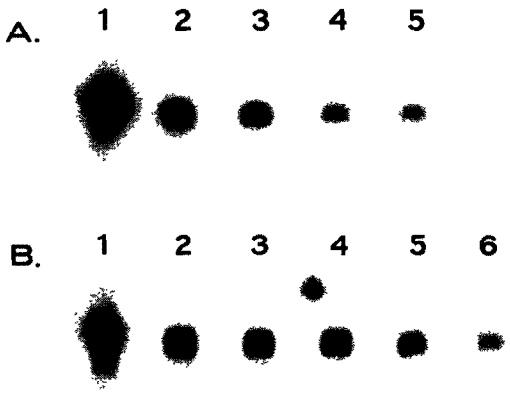

Fig. 2. Modulation of protein tyrosine phosphatase activity in HER14 cells by PMA and EGF. Serum-starved HER14 cells were incubated for fifteen min with increasing concentrations of PMA (A) (0.01, 0.1 and 1 μM in lines 3, 4 and 5, respectively), or with 1 μM PMA for increasing time periods (B) (1, 3, 15 and 30 min, lines 3 through 6, respectively). Control cells (line 2 in A and B) received DMSO (0.2%). Reaction was terminated and homogenate was prepared to measure protein tyrosine phosphatase activity on ^{32}P-labeled EGF-R as a substrate (see Section 2). Line 1 (A,B) refers to radioactive EGF-R incubated with buffer A alone. The inset (see page 16 of this manuscript) shows data in cpm (mean ± S.E.M. of five experiments) of radioactivity associated with ^{32}P-labeled EGF-R when incubated with buffer A alone, or with cell homogenate derived from cells that have been treated for 15 min with either DMSO or PMA. DMSO and PMA treatments are compared using two tail Student's t-test.

Buffer	16,142 ± 2,383
DMSO	10,445 ± 2,507
PMA	6,578 ± 1,767

$P < 0.02$.

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