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Inhibition of stimulus-dependent epidermal growth factor receptor and transforming growth factor-α mRNA accumulation by the protein kinase C inhibitor staurosporine

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The ability of staurosporine, a potent inhibitor of protein kinase C, to block certain cellular events initiated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor (EGF) was examined. Treatment of MDA468 breast cancer cells with TPA decreases EGF binding to the cell surface and this effect is blocked by pretreatment with staurosporine with an IC_{50} of 30 nM. Either 10^{-9} M EGF or 100 ng/ml TPA stimulated the accumulation of both EGF receptor and TGF-α mRNA and staurosporine (50 nM) completely abolished these mRNA accumulations. Staurosporine did not block EGF-stimulated tyrosine phosphorylation of its receptor as measured by immunoblotting with anti-phosphotyrosine antibodies. The ability of staurosporine to block the mRNA responses of either EGF or TPA suggests that these two agents have common signaling pathways and it implies a role for protein kinase C in the control of EGF receptor and TGF-α expression.

Growth factor receptor; Protein kinase C; Tumor promotor; Phorbol ester

1. INTRODUCTION

Protein kinase C is a Ca^{2+}/phospholipid-dependent enzyme that is activated directly by diacylglycerol generated during receptor-mediated inositol phospholipid turnover [1,2] or by tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [3–5]. Activation of protein kinase C is known to have a variety of effects on cells (for review, see [6]) and is thought to play important roles in both signal transduction and cellular proliferation. In MDA468 cells, we have utilized TPA to examine the effects of protein kinase C activation on epidermal growth factor (EGF) receptor and transforming growth factor-α (TGF-α) expression and have found that TPA stimulates the accumulation of both EGF receptor and TGF-α mRNA. We have also shown that EGF can stimulate EGF receptor and TGF-α mRNA accumulation and have previously postulated that protein kinase C may have an important role in these actions of EGF [7]. To further characterize the role of protein kinase C in EGF action, we have employed the recently described protein kinase C antagonist, staurosporine [8], and have found that it blocks the stimulation of EGF receptor and TGF-α expression induced by both TPA and EGF. This finding is compatible with our earlier hypothesis that the protein kinase C is a key enzyme in mediating the effects of TPA and EGF on EGF receptor and TGF-α expression in the MDA468 human breast cancer cell line.

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2. MATERIALS AND METHODS

2.1. *Materials*

Staurosporine (from Kyowa Hakko Kogyo, Japan) was dissolved in dimethyl sulfoxide. Mouse monoclonal antibody B1D8 against the EGF receptor was prepared as in [9]. Rabbit

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anti-phosphotyrosine antibodies were prepared and purified as described by Kamps and Sefton [10].

2.2. *Cell cultures*

MDA468 cells were maintained in a CO_2 incubator on 15-cm Falcon tissue culture plates in Dulbecco's modified Eagle's medium containing 10% calf serum plus penicillin and gentamicin.

2.3. *EGF-binding studies*

EGF binding was quantitated as described [7] using ^{125}I-EGF equivalent to 8×10^{-11} M EGF (30000 cpm/well).

2.4. *RNA preparation and hybridization*

RNA was prepared by the method of Chirgwin et al. [11]. 20 μg total RNA per lane was electrophoresed in 1% agarose, 2.2 M formaldehyde gels [12]. The RNA was transferred to nitrocellulose, prehybridized, and hybridized with the appropriate [^{32}P]cDNA probe (EGF receptor [13]; TGF-α [14]) labeled [15] to a specific activity of 2.0×10^9 dpm/μg. Unbound probe was washed off at high stringency [16].

2.5. *Elution of hybridized probes*

The blot was washed in 500 ml of 2 mM Tris, 2 mM EDTA, 0.1% SDS (pH 8.2) for 20 min at 65°C [17].

2.6. *Measurement of phosphotyrosine incorporation into the EGF receptor*

The MDA468 cells were rinsed twice with ice-cold PBS, and lysed with 2.0 ml ice-cold phosphate-RIPA buffer containing 2 mM EDTA and 1 mM sodium orthovanadate as described by Kamps and Sefton [10]. The samples were thoroughly mixed by vortex-mixing and spun at 13000 × *g* for 15 min at 4°C to remove insoluble material. 200 μl of the supernatant was removed and 20 μg of the anti-EGF receptor monoclonal antibody B1D8 was added. After incubation for 2 h at 4°C, 100 μl fixed *S. aureus* (Pansorbin, Calbiochem) was added and incubated for 30 min. The bacterial complex was then centrifuged and washed twice with the cell-lysing buffer, resuspended and boiled in Laemmli sample buffer, and the supernatant electrophoresed on a 7.5% SDS-polyacrylamide gel [18]. The protein in the gel was then electrophoretically transferred to nitrocellulose [10] followed by incubation of the blot overnight at 4°C in blocking buffer [10]. The blot was incubated in blocking buffer containing 0.75 μg/ml affinity-purified phosphotyrosine-specific rabbit antibodies for 2 h and then washed twice for 10 min in blocking solution. The wash was followed by incubation with 200 nCi/ml of ^{125}I-protein A for 1 h, a final wash as before, and autoradiography of the blot.

3. RESULTS

To test the ability of staurosporine to block a well-characterized protein kinase C-mediated event, we examined whether pretreatment with staurosporine for 2 h (to allow partitioning and enzyme inhibition) could block the ability of TPA to decrease the number of high-affinity EGF receptor binding sites on the cell surface of MDA468 cells. Fig.1 shows that cells treated with TPA alone undergo a decrease in the binding of ^{125}I-EGF. We have previously shown that this loss of binding results from a loss of high-affinity binding sites in these cells [7]. Beginning at 20 nM, staurosporine caused a dose-dependent inhibition of the effects of TPA on EGF binding, with an estimated IC_{50} value of approx. 30 nM. The highest dose of staurosporine routinely used (80 nM) blocked 87% of the effect of TPA. This inhibition occurred in the absence of any significant effect of staurosporine on EGF binding in control cells and in the absence of any observable cytotoxicity.

Next, we investigated whether staurosporine could block the ability of EGF or TPA to cause an accumulation of EGF receptor and TGF-α mRNA. The MDA468 cells were pretreated with 50 nM staurosporine, a concentration shown in the previous experiment to block the majority of TPA's effects on EGF binding. Fig.2 shows that in the absence of staurosporine, both EGF and TPA cause an accumulation of both EGF receptor and

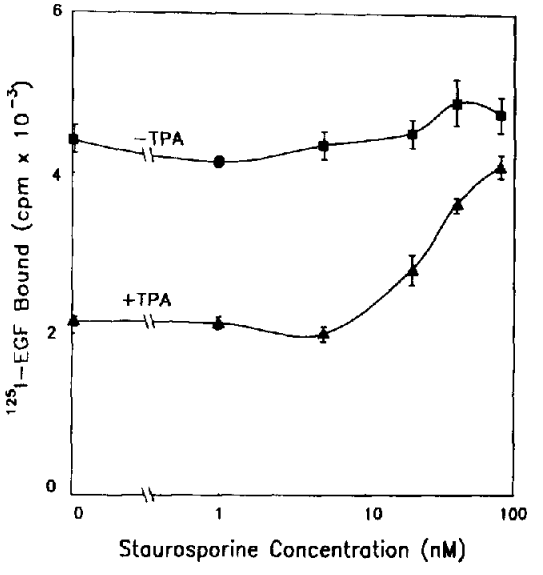

Fig.1. Effect of staurosporine on the loss of EGF binding caused by TPA. MDA468 cells were treated with the indicated doses of staurosporine in 0.5 ml of medium for 2 h, followed by treatment in the absence (■) or presence (▲) of 100 ng/ml of TPA for a further 2 h. EGF binding was then measured. Non-specific binding of radioactive EGF as assessed by incubation in the presence of 2×10^{-7} M unlabeled EGF was always below 0.5% of the total counts added. Results represent means ± SE of triplicate wells.

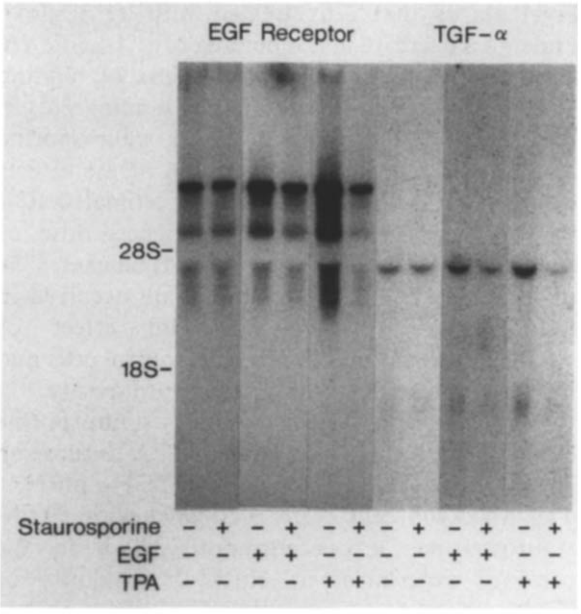

Fig.2. Effect of staurosporine on the EGF- or TPA-induced accumulation of EGF receptor and TGF-α mRNA. MDA468 cells were treated in the absence or presence of 50 nM staurosporine for 2 h, followed by incubations in the presence of either 10^{-9} M EGF or 100 ng/ml TPA for a further 7 h. Cellular mRNA was probed with the TGF-α cDNA probe. Following autoradiography, the TGF-α probe was stripped from the membrane and the blot reprobed with the EGF receptor cDNA probe.

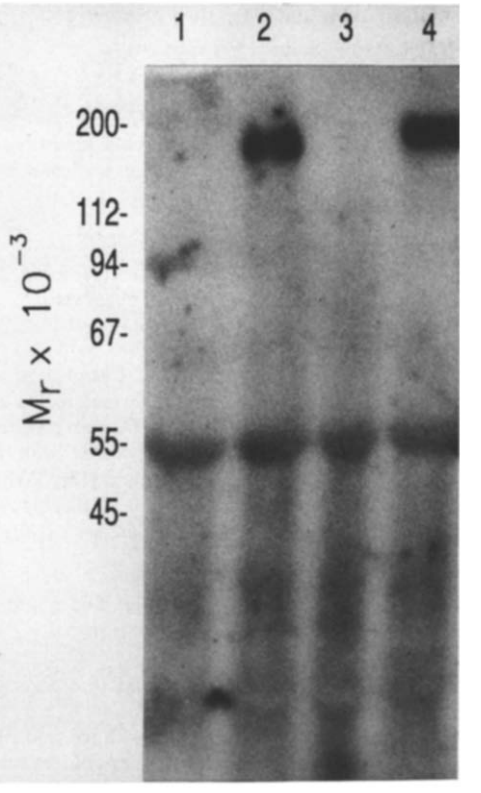

Fig.3. Effect of staurosporine on EGF-stimulated tyrosine phosphorylation of the EGF receptor. MDA468 cells were treated in the absence (lanes 1,2) or presence (lanes 3,4) of 50 nM staurosporine for 2 h, followed by incubations in the absence (lanes 1,3) or presence (lanes 2,4) of 10^{-9} M EGF for 15 min. The EGF receptor was immunoprecipitated using an anti-EGF receptor antibody and immunoblotted with an anti-phosphotyrosine antibody. The quantity of bound antibody was determined with ^{125}I-protein A followed by autoradiography.

TGF-α mRNA. In the presence of staurosporine, the effects of both EGF and TPA on EGF receptor and TGF-α mRNA are blocked. Staurosporine had no significant effect on either mRNA level in control cells. Blots stripped and reprobed with the 'housekeeping probe' hexosaminidase A confirmed the absence of RNA gel loading artifacts and the relative specificity of the response (not shown).

Because staurosporine could block the effects of EGF on EGF receptor and TGF-α mRNA accumulation, we examined if this inhibitory effect could have been mediated through direct effects on the intrinsic tyrosine kinase activity of the EGF receptor. The MDA468 cells were pretreated for 2 h with 50 nM staurosporine, followed by a 15 min EGF treatment and measurement of the level of phosphotyrosine in immunoprecipitated EGF receptor. Fig.3 shows that both in the absence and presence of staurosporine, EGF stimulated phosphorylation of its receptor on tyrosine residues, with no obvious inhibitory effects displayed by staurosporine.

4. DISCUSSION

Staurosporine has been found to be a non-competitive inhibitor of protein kinase C with respect to phospholipid, Ca^{2+}, protein substrate (histone), ATP, diacylglycerol and phorbol esters [8]. The present study describes the ability of staurosporine to block certain cellular responses in the MDA468 breast cancer thought to be mediated by protein kinase C. The activation of protein

kinase C by TPA in the MDA468 cells, as in many others, results in the loss of high-affinity EGF-binding sites [19,20]. This effect of TPA is believed to be mediated through phosphorylation of the EGF receptor at Thr 654 by protein kinase C [21–23]. Pretreatment of the cells with staurosporine resulted in a dose-dependent diminution in the effect of TPA on EGF binding, implying that staurosporine indeed blocked TPA-stimulated EGF receptor transmodulation.

Having established that staurosporine was capable of inhibiting a well characterized effect of protein kinase C activation in intact MDA468 cells, we examined whether staurosporine could block the more distal effect of TPA on the expression of the EGF receptor and TGF-α in these cells. In the absence of staurosporine, TPA stimulated the accumulation of both the EGF receptor and TGF-α mRNAs, while in its presence, this effect of TPA was completely blocked. EGF is also capable of stimulating EGF receptor [24,25] and TGF-α mRNA [26] accumulation in certain cells including the MDA468 cells. Staurosporine also blocked these EGF-stimulated effects. Previously, using TPA to downregulate protein kinase C in MDA468 cells, we could block the stimulation of EGF receptor synthesis by TPA and EGF [7]. We hypothesized that the effect of EGF on the expression of the EGF receptor requires protein kinase C. The ability of staurosporine to block EGF-stimulated expression of the EGF receptor and TGF-α supports this hypothesis.

While staurosporine inhibits protein kinase C activity, it may also inhibit the activity of other protein kinases [8,27] involved in signal transduction, such as the intrinsic protein tyrosine kinase of the EGF receptor. The activity of this protein kinase is necessary for EGF action [28] and its inhibition by staurosporine could explain the blockade of EGF-stimulated events. Autophosphorylation of the EGF receptor is an early step in EGF signaling which can increase the affinity of the protein tyrosine kinase for other substrates [29]. Thus, EGF receptor autophosphorylation was examined to determine if staurosporine could block EGF action at this level. Pretreatment of intact cells with sufficient staurosporine to block TPA and EGF effects had no detectable effect on EGF-stimulated receptor autophosphorylation. Since autophosphorylation of the receptor was unaffected by staurosporine, it is improbable that staurosporine blocked distal effects of EGF by blocking the EGF receptor protein tyrosine kinase. A more plausible explanation, combined with our earlier results in protein kinase C-depleted cells, would be that EGF requires activatable protein kinase C for its action on the expression of the EGF receptor and TGF-α. In concert with this notion, EGF has been shown to stimulate the accumulation of inositol trisphosphate (IP_3) in MDA468 cells [30] suggesting that EGF stimulation might involve second messenger generation via the phosphatidylinositol cycle. Furthermore, evidence that the EGF receptor may phosphorylate phospholipase C and activate this enzyme [31] may explain the mechanism by which protein kinase C is involved in EGF action. The ability of staurosporine to block responses to both EGF and TPA suggests that these two agents signal through common pathways.

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