Vol. 86, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1979 Pages 1037-1043

INHIBITION OF EPIDERMAL GROWTH FACTOR BINDING TO SURFACE RECEPTORS BY TUMOR PROMOTORS

KENNETH D. BROWN, PHILLIP DICKER and ENRIQUE ROZENGURT

Imperial Cancer Research Fund, Lincoln's Inn Fields
London WC2A 3PX, U.K.

Received January 2, 1979

SUMMARY

The tumor promotor, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), causes a marked (about 10-fold) decrease in the affinity of epidermal growth factor (EGF) receptors for [^{125}I]-EGF. The inhibition exhibits specificity, dependence on temperature, and persistence after removal of the promotor. TPA does not induce "down-regulation" of EGF receptors. Finally, the promotor interacts synergistically with EGF in stimulating glycolysis and DNA synthesis in 3T3 cells. The results suggest that TPA does not bind directly to EGF receptors.

12-O-tetradecanoyl-phorbol-13-acetate (TPA)* is a potent tumor promotor in carcinogen initiated mouse skin. The compound stimulates a complex set of biochemical events in cultured cells (1) including DNA synthesis and cell division (2,3). Recently, we found that TPA stimulates DNA synthesis in the absence of added serum interacting synergistically with polypeptide hormones like epidermal growth factor (EGF), insulin, and the growth factor purified from the medium conditioned by SV40 BHK cells (4). These findings prompted us to investigate the effect of TPA on the binding of radiolabeled EGF to surface receptors. We found that TPA is a potent inhibitor of the binding of [^{125}I]-EGF to Swiss 3T3 cells. Lee and Weinstein (5) have recently reported that TPA reduces the number of binding sites available to EGF in cultures of HeLa cells. In contrast, our findings show that TPA causes a large decrease in the affinity of the cellular receptors for EGF without changing the total number of binding sites. We also show that the mechanisms of inhibition of [^{125}I]-EGF binding by TPA and native EGF are different which suggests that the tumor promotor does not bind directly to EGF receptors.

MATERIALS AND METHODS

Cell cultures. Swiss mouse 3T3 cells, propagated as previously described (6), were subcultured into 33mm Nunc dishes in Dulbecco-Vogt modified Eagle's medium

*Abbreviations: TPA: 12-O-tetradecanoyl-phorbol-13-acetate. EGF: epidermal growth factor. [^{125}I]-EGF: [^{125}I]-labeled epidermal growth factor. DME: Dulbecco-Vogt modified Eagle's medium. BSA: bovine serum albumin. BES: N,N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid.

0006-291X/79/041037-07$01.00/0

(DME) with 10% fetal bovine serum. The cells were re-fed after four days' growth and incubated for at least a further four days before use in the assays.

Epidermal growth factor and [^{125}I]-labeled EGF ([^{125}I]-EGF). EGF was prepared from mouse salivary glands by the method of Savage and Cohen (7). The soluble lactoperoxidase procedure (8) was used to label the EGF with [^{125}I]. The labeled protein was separated from unreacted Na-[^{125}I] by passage of the iodination mixture through a Sephadex G-25 column equilibrated and eluted with a buffer containing 0.01M-phosphate, pH 7.4 and 0.15M-sodium chloride. The labeled EGF was stored frozen in the presence of 0.1% bovine serum albumin (BSA). The specific activity of the [^{125}I]-EGF, at preparation, was 140,000-170,000 cpm/ng.

[^{125}I]-EGF binding assay. The growth medium was removed from the dish and the cells were washed twice with 1-2ml of binding medium which consisted of DME containing 0.1% BSA, 10^{-7}M potassium iodide, 50mM N,N-bis-(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES) adjusted to pH 6.8. The cells were incubated at 37°C or 0°C (ice bath) with 1ml of binding medium containing [^{125}I]-EGF at the required concentration. After 90 min, unbound radioactivity was removed by washing the cells four times with cold (4°C) phosphate-buffered saline (pH 7.4). The cells were extracted (60 min at 37°C) with 1ml of 0.5N NaOH and cell-associated radioactivity was determined in a gamma counter.

Non-specific binding, determined as the amount of radioactivity bound in the presence of 2µg/ml of unlabeled EGF, was substracted from all of the results. Non-specific binding was always less than 5% of the total.

Measurement of DNA synthesis and glycolysis. DNA synthesis and glycolysis were assayed as described previously (6,9).

RESULTS

Unlabeled EGF and TPA produced potent, dose-dependent inhibition of [^{125}I]-EGF binding to 3T3 cells (Fig. 1). In contrast, three compounds structurally related to TPA, but with much reduced biological activity (1,10), did not inhibit [^{125}I]-EGF binding even at concentrations as high as 1µg/ml (Fig. 1). TPA did not affect the binding of [^{125}I]-insulin to 3T3 cells (unpublished results). The results presented in Fig. 2 indicate that the inhibition of [^{125}I]-EGF binding by TPA is competitive with no change in maximal binding capacity (Bmax), but with a large decrease (about 10-fold) in the apparent affinity of binding. In addition, three separate experiments (not shown) demonstrated that, at 25ng/ml [^{125}I]-EGF, the inhibition of binding by 100ng/ml TPA was completely abolished.

A number of experiments indicate that the mechanisms of inhibition of [^{125}I]-EGF binding by TPA and native EGF are not identical, and suggest that TPA does not bind directly to EGF receptors. EGF inhibits [^{125}I]-EGF binding measured at 37°C or 0°C whereas TPA is inhibitory at 37°C but not at 0°C (Figs. 1 and 3). The inhibitory effect of EGF is rapidly reversed when EGF is removed from the binding medium whereas the effect of TPA persists after removal of the promotor (Fig. 4). An interesting finding is that the persistence of the effect of TPA on [^{125}I]-EGF binding at 37°C is evident in cells exposed to the promotor at 0°C (Table 1). Thus, at 0°C TPA becomes associated with 3T3 cells but does not inhibit [^{125}I]-EGF binding.

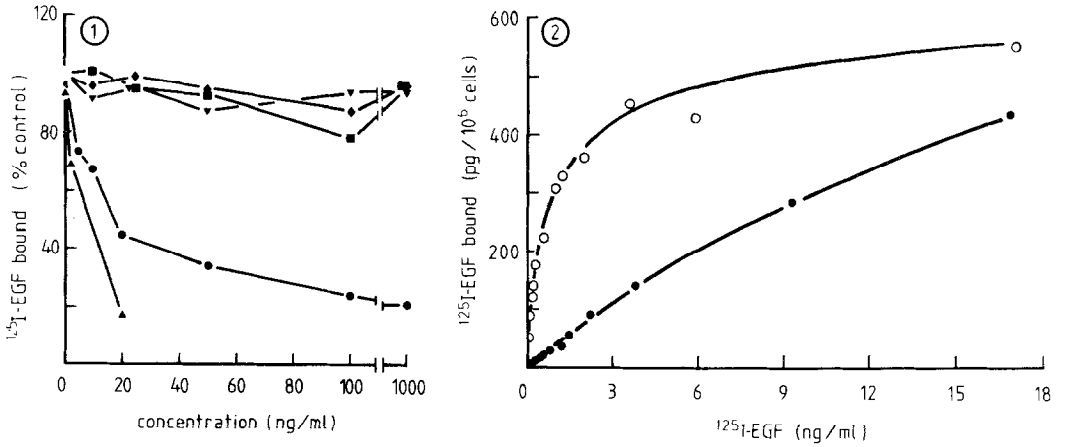

Fig. 1. Effect of EGF, TPA and TPA-related compounds on the binding of [^{125}I]-EGF to 3T3 cells. One milliliter of binding medium (37°C) containing 2ng/ml of [^{125}I]-EGF (120,000 cpm/ng) and the test compounds at the indicated concentrations was added to dishes of cells. The dishes were incubated at 37°C for 90 min, washed, and the cell-associated radioactivity was determined as described under Materials and Methods. Each point represents the mean value (n = 3) for EGF binding expressed as a percentage of the mean control value of 241 ± 5 (SE) pg/10^6 cells (10,980 cpm/dish). ●, TPA; ▲, EGF; ◆, phorbol; ■, 4-O-methyl-12-O-tetradecanoyl-phorbol-13-acetate; ▼, phorbol-12,13,20-triacetate.

Fig. 2. Effect of [^{125}I]-EGF concentration on binding to 3T3 cells in the presence or absence of TPA. Various concentrations of [^{125}I]-EGF (0.1 - 17ng/ml, 170,000cpm/ng) in 1ml of binding medium, with (●) or without (○) TPA (100ng/ml), were added to dishes. The cell-associated radioactivity was determined after a 90-min incubation at 37°C. Each point represents the value for EGF binding obtained from a single dish of cells.

Table 1. Persistence of inhibition of [^{125}I]-EGF binding at 37°C after exposure of cells to TPA at 0°C

Binding temperature (°C)	[^{125}I]-EGF bound per 10^6 cells (pg)	
	Pretreatment addition	
	none	100ng/ml TPA
0	66 ± 3	62 ± 1
37	171 ± 5	50 ± 4

Cells were rinsed once with binding medium at 0°C. One milliliter of binding medium (0°C) with or without TPA was added per dish and the dishes were incubated at 0°C for 60 min. The cells were rinsed four times with binding medium. Cell-associated radioactivity was measured after a 90-min incubation at 0°C or 37°C with 1ml of binding medium containing 1ng/ml [^{125}I]-EGF (104,000cpm/ng). The mean value (± S.E.) of three determinations of [^{125}I]-EGF binding is shown. The mean values (± S.E.) for [^{125}I]-EGF binding when TPA (100ng/ml) was present in the binding medium were 61 ± 3 pg/10^6 cells at 0°C and 18 ± 1 pg/10^6 cells at 37°C.

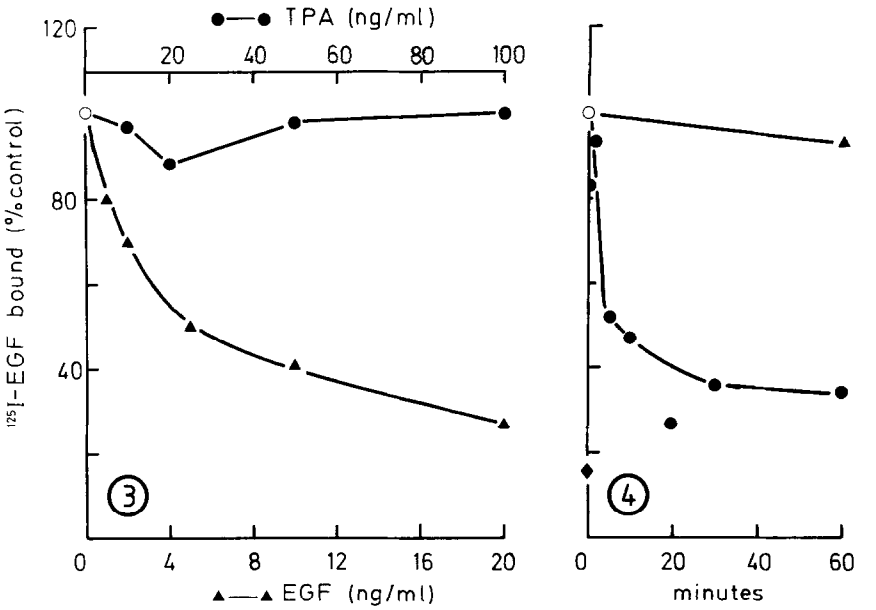

Fig. 3. Inability of TPA to inhibit [^{125}I]-EGF binding to 3T3 cells at 0°C. Cell-associated radioactivity was determined after a 90-min incubation at 0°C with 1ml of binding medium containing 2ng/ml [^{125}I]-EGF (92,000cpm/ng) and TPA (●) or unlabeled EGF (▲) at the indicated concentrations. Each point represents the mean value for EGF binding (n = 3) expressed as a percentage of the mean control value of 113 ± 6 (S.E.) pg/10^6 cells.

Fig. 4. Persistence of TPA-mediated inhibition of [^{125}I]-EGF binding at 37°C. Cells were rinsed once with binding medium. One milliliter of binding medium (37°C) was added per dish. At intervals, 10µl aliquots of TPA (●) or EGF (▲) were added to the dishes to give final concentrations of 100 and 10ng/ml respectively. During this addition the medium was gently swirled in the dishes to promote rapid mixing. After 60 min, all dishes were washed four times with binding medium and 1ml of binding medium containing 1ng/ml [^{125}I]-EGF (122,000 cpm/ng) was added per dish. The cell-associated radioactivity was determined after a 90-min incubation at 37°C. Each point represents the mean value (n=2) for EGF binding expressed as a percentage of the mean (n = 3) control value of 180 pg/10^6 cells. The mean value for EGF binding in the presence of 100ng/ml TPA is shown (◆).

If TPA inhibits EGF binding by occupying EGF receptors, pretreatment of cells with saturating concentrations of EGF should reduce or eliminate the persistent inhibition of binding produced by TPA. The results presented in Table 2 show that this is not the case. Indeed, the inhibition of binding was found to be 52% and 63% in pretreated and non-pretreated cells respectively, suggesting that TPA and EGF interact with different binding sites.

Exposure of cells to EGF for several hours reduces the number of EGF binding sites (Bmax) on the plasma membrane (11,12). This "down-regulation" is due to

Table 2. Effect of pretreatment with saturating concentrations of EGF on the inhibition of [^{125}I]-EGF binding by TPA

Pretreatment additions			[^{125}I]-EGF bound per 10^6 cells (pg)
90 min	-	45 min	
none	-	none	139 ± 5
none	-	TPA	52 ± 7
EGF	-	none	113 ± 2
EGF	-	TPA	54 ± 12

Cells were rinsed once with binding medium at 0°C. One milliliter of binding medium with or without 50ng/ml of EGF was added per dish and the dishes were incubated at 0°C for 90 min. A 10µl aliquot of TPA was added to some dishes to give a final concentration of 100ng/ml. All dishes were incubated at 0°C for a further 45 min. The dishes were washed four times with binding medium at 37°C. Cell-associated radioactivity was measured after a 90-min incubation at 37°C with 1ml of binding medium containing 1ng/ml [^{125}I]-EGF (71,000 cpm/ng). The mean value (± S.E.) of four determinations of [^{125}I]-EGF binding is shown.

endocytic internalization of occupied EGF receptors (13,14). Fig. 5 shows that 8 hours of treatment of 3T3 cells with concentrations of TPA as high as 1µg/ml did not reduce the Bmax while treatment with 2ng/ml EGF caused a 50% decrease in its value. We conclude that TPA, in contrast to EGF, does not induce "down-regulation" of EGF receptors.

The conclusion that TPA and EGF interact with different binding sites is further supported by experiments in which these agents are shown to trigger biological responses in a synergistic manner. Fig. 6 shows that TPA acts synergistically with EGF in stimulating glycolysis and DNA synthesis in quiescent cells. That EGF can initiate a biological response when EGF binding is inhibited (in the presence of TPA) is presumably due to the availability of "spare receptors" (15) for the growth factor. It is known that the maximal response to EGF can be obtained at concentrations at which only a small fraction of EGF receptors is occupied (12,16). Synergism would not be expected if the compounds were directly interacting with the same receptor-effector system.

DISCUSSION

Several characteristics of TPA activity suggest that the promotor acts through specific membrane receptors (17). TPA is biologically active at low concentrations. Furthermore, the various effects of TPA exhibit stereospecificity and saturability. Our results show that TPA is a potent, competitive inhibitor of [^{125}I]-EGF binding to 3T3

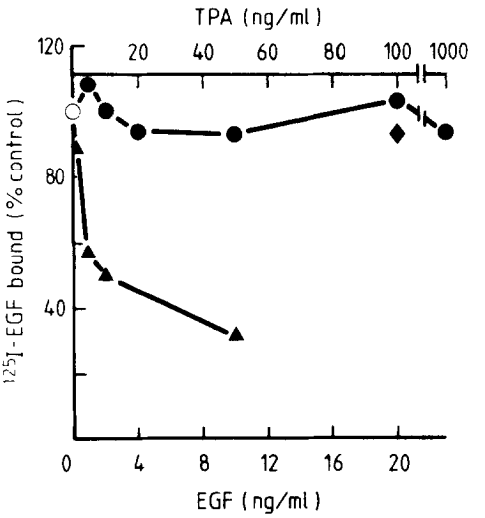

Fig. 5. Inability of TPA to induce "down-regulation" of EGF binding sites. One milliliter of binding medium containing the indicated concentrations of TPA (●) or EGF (▲) was added to dishes of cells. The dishes were incubated for 8 hours at 37°C. The cells were rinsed twice with binding medium. Cell-associated radioactivity was determined after a 90-min incubation at 37°C with 1ml of binding medium containing 24ng/ml [^{125}I]-EGF (141,000 cpm/ng). Each point represents the mean value (n = 3) for EGF binding expressed as a percentage of the mean control value. The mean value for EGF binding in the presence of 100ng/ml TPA is shown (◆).

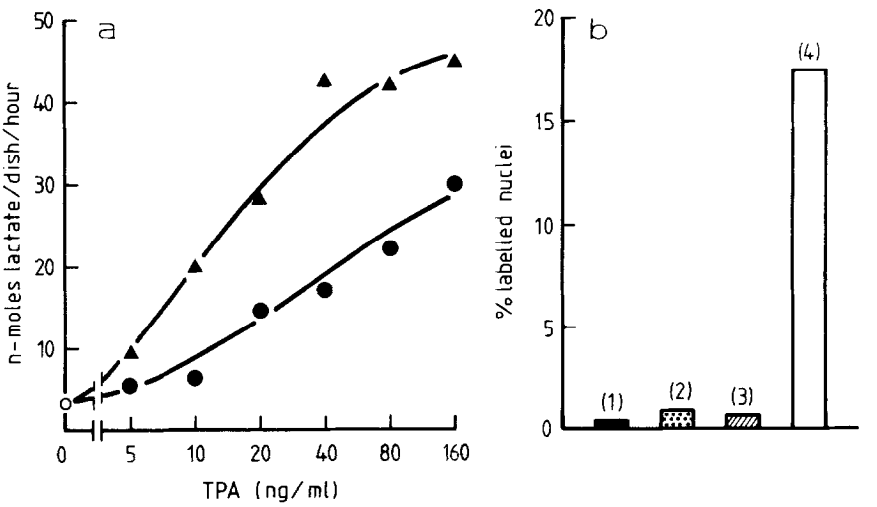

Fig. 6. Synergistic stimulation of glycolysis (a) and DNA synthesis (b) by TPA and EGF in 3T3 cells. Panel a: Lactic acid production was measured (9) during a 4-hour exposure of cells to medium containing the indicated concentration of TPA, with (▲) or without (●) 1ng/ml EGF. Each point represents the mean value obtained from 2 dishes of cells. Panel b: The percent of radioactively labeled cell nuclei was determined (6) after a 40-hour exposure to 2ml of a 1:1 mixture of DME and Waymouth's medium (19) containing [^{3}H]-thymidine (5μCi/ml; 0.2μM) and (1) no other additions, (2) 100ng/ml TPA, (3) 10ng/ml EGF, (4) 100ng/ml TPA + 10ng/ml EGF. The mean value from duplicate determinations is shown.

cells. This, and the findings of Lee and Weinstein (5), raises the possibility that the diverse biological effects of TPA are mediated by the EGF receptor-effector system. However, the inhibition of [^{125}I]-EGF binding by TPA differs in several important characteristics from the inhibition of binding produced by native EGF. Furthermore, TPA does not induce "down-regulation" of EGF receptors and the promotor acts synergistically with EGF in stimulating glycolysis and DNA synthesis. In addition, it has been reported that cells lacking EGF receptors are responsive to TPA (18).

Taken together, these findings suggest that TPA initially binds to sites which are separate from EGF receptors and that TPA-occupied sites subsequently interact with the EGF receptor thereby reducing their affinity for EGF in a temperature-sensitive fashion. The proposed interaction between TPA and EGF receptors has important implications. Firstly, it suggests a novel way in which cellular binding of growth factors and hormones could be modulated. Secondly, it offers a system to characterize further the mechanism of action of tumor promotors.

Acknowledgments. We thank Patricia Pettican and Ann Legg for the preparation of cell cultures.

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