Vol. 98, No. 3, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1981 Pages 829-835

CALMODULIN INHIBITORS MODIFY CELL SURFACE CHANGES TRIGGERED BY A TUMOR PROMOTER.

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Received December 29,1980

Summary: Trifluoperazine (TFP) and other inhibitors of the Ca^{2+}- binding protein calmodulin, modified two early responses of cultured mammalian cells to the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA). In the presence of 40μM TFP mouse epidermal cells were insensitive to the TPA-inhibition of epidermal growth factor binding. TFP also caused a marked inhibition of the basal rate of [^{3}H]choline incorporation into HeLa cell phospholipids, and largely overcame the TPA stimulation of choline incorporation.

Tumor promoters induce a wide range of biochemical changes in cultured mammalian cells, many of which are associated with the cell surface (see 1 for references). Proposals linking these changes to the action of promoters have been strengthened by the identification of specific membrane binding sites for promoters (2-4).

There is little firm information on the mechanisms involved in the generation of multiple membrane-associated changes following the binding of promoters to the cell surface. In the present work we explore the possibility that Ca^{2+} may be involved in some of the promoter-induced changes. Use has been made of the phenothiazine antipsychotic agents which bind strongly to the Ca^{2+}- binding protein calmodulin, thereby inactivating it (5). It is now known that many of the biological effects of Ca^{2+} are mediated via calmodulin (6). There is already evidence that some of the surface changes triggered by promoters involve Ca^{2+} (7-10). For example, the tumor promoter TPA has been shown to stimulate the release of

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Abbreviations: DMSO, dimethyl sulfoxide; TPA, 12-0-tetradecanoylphorbol-13-acetate; TFP, trifluoperazine; EGF, epidermal growth factor; MEM, Eagles minimal essential medium.

0006-291X/81/030829-07$01.00/0

arachidonic acid from membrane phosphoglycerides (9, 10), a process catalysed by phospholipase A_2 (11). Phospholipase A_2 is a Ca^{2+}- dependent enzyme (11) and the Ca^{2+} requirement is probably mediated by calmodulin (12).

In this initial study, the effects of calmodulin inhibitors on the following early effects of TPA have been examined:

1. Stimulation of [^{3}H]choline incorporation into membrane phospholipids (13).
2. Inhibition of the binding of EGF to cellular receptors (14-20).

MATERIALS AND METHODS.

Materials: The mouse epidermal cell line (HEL-37; used at passages 185-190) was maintained as described before (21). HeLa cells were maintained in a similar way. TPA was obtained from PL Biochemicals Inc., Milwaukee, Wis., U.S.A. The preparation and specific activity of [^{125}I]EGF were as described before (17,18). [methyl^{3}H]Choline (sp. act. 77 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, U.K. TFP and haloperidol were gifts from Smith Kline and French Laboratories, Australia and Searle Australia Pty. Ltd. respectively. Both penfluridol and pimozide were gifts from Janssen Pharmacentica Pty. Ltd., N.S.W., Australia. Chlorpromazine and tetracaine were obtained from Sigma Chemicals, St. Louis, U.S.A.

[^{125}I]EGF-binding assays. Binding to HEL-37 cells was measured at 30^o or 2^{o}C as described before (17,18). Assays were carried out 3-4 days after plating, a time at which the cells were approaching confluence. Binding data is given as specific EGF bound (total [^{125}I]EGF bound minus [^{125}I]EGF bound in the presence of a 160-fold excess of unlabelled EGF). All binding assays contained 1nM EGF. Assays containing TFP were preincubated for 30 min with the drug before [^{125}I]EGF was added.

[^{3}H]Choline incorporation. HeLa cells (0.6×10^6) were plated on 6 cm dishes, and used after 2 days ($2 - 4.5 \times 10^6$ cells per dish). The cells were incubated with fresh MEM (2 ml) containing 0.8µCi [methyl^{3}H]choline together with the test substance in DMSO or with DMSO alone. After appropriate incubation times at 37^o, the medium was removed and the cells washed twice with cold phosphate-buffered saline (pH 7.4). The cells were collected in 4 ml cold 4% perchloric acid. The suspension was centrifuged, the pellet suspended in 3 ml of chloroform-methanol (2 : 1, *v/v*) and the tubes stirred vigorously for 15 sec. After addition of 1 ml of 0.5 M KCl in methanol : H_2O (1 : 1; *v/v*) to each tube, the tubes were again stirred for 10 sec. After 30 min the chloroform layer was carefully removed, again washed with KCl-methanol, and finally transferred to a scintillation vial. The chloroform was removed by evaporation and radioactivity measured by liquid scintillation counting.

RESULTS AND DISCUSSION.

The effect of TFP on the inhibition of [^{125}I]EGF binding to HEL-37 cells by TPA is shown in Fig. 1A. At concentrations of between 25-40 µM TFP, EGF binding in the absence of TPA was inhibited by about 60%; at these concentrations of TFP, EGF binding was not depressed further by including TPA in

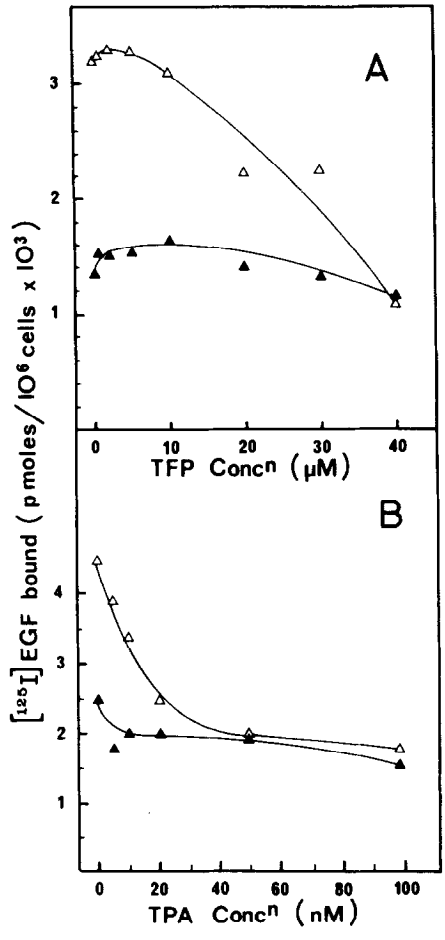

FIGURE 1. Effect on TFP and TPA on [^{125}I]EGF binding to HEL-37 cells.
A. Effect of TFP concentration on EGF binding in the absence (△) and presence (▲) of 100 nM TPA.
B. Effect of TPA concentration on EGF binding in the absence (△) and presence (▲) of 25 μM TFP.

the binding assay. Similarly in the presence of 25 μM TFP, EGF binding was relatively insensitive to a range of TPA concentrations (Fig. 1B). At concentrations of TFP up to 50 μM, the viability of the HEL-37 cells was greater than 95% over the assay period (as measured by trypan blue exclusion). At higher TFP concentrations, cell viability decreased, and specific [^{125}I]EGF binding both in the presence and absence of TPA also decreased markedly (data not shown).

TABLE 1. Effect of TPA and TFP on [^{125}I]EGF binding to HEL-37 cells at 30°C and 2°C.

Treatment	[^{125}I]EGF bound (pmoles/10^6 cells x 10^{-3})			
	30°C	% control	2°C	% control
DMSO	3.01±0.13	100	2.49±0.10	100
100 nM TPA	1.32±0.05	44	2.14±0.07	86
25 µM TFP	2.35±0.12	78	1.64±0.10	66
100 nM TPA +25 µM TFP	1.52±0.04	50	1.79±0.07	72

Binding assays were carried out as described in Materials and Methods except that assays at 2° were incubated for 180 min. Values are the mean ± S.E. of 5 separate determinations.

The partial inhibition of [^{125}I]EGF binding by TFP is similar to that observed with TPA (18). However unlike TPA, the TFP inhibition could not be reversed by increasing the concentration of EGF (data not shown), and also occurred when binding assays were carried out at 2°C (Table 1). Thus 25 µM TFP inhibited EGF binding by 22% and 34% when assays were carried out at 30° and 2° respectively; corresponding inhibitions by 100 nM TPA were 56% and 14%. Possible mechanisms for the TFP effect include differential inhibition of EGF binding to a sub-class of EGF receptors also sensitive to TPA inhibition (20) or an uncoupling of EGF receptors from intramembrane interactions in a manner similar to that proposed for TPA (18).

As shown in Fig. 2, TFP also markedly inhibited both the basal and the TPA-stimulated rate of [methyl^{3}H]choline incorporation into the phospholipids of HeLa cells. Again, 25 µM TFP did not affect the viability of HeLa cells tested after 2 h incubation. The mechanism for the inhibition is not known. However, inhibition of a Ca^{2+}- dependent phospholipase A_2 is a possibility if, for example, an arachidonic acid metabolite is involved in the TPA stimulation of choline incorporation into phospholipid (13). It is also possible that TFP inhibited the incorporation of [^{3}H]choline into the acid-soluble pool.

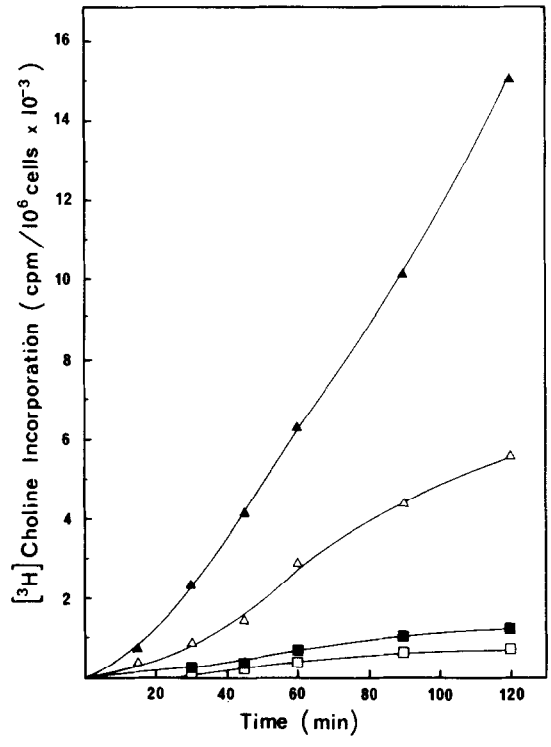

FIGURE 2. Effect of TPA and TFP on the incorporation of [methyl ^{3}H] choline into HeLa cell phospholipids. Assays (carried out in triplicate) contained DMSO (△), 0.1 μM TPA (▲), 25 μM TFP (□) or 0.1 μM TPA + 25 μM TFP (■).

A number of antipsychotic agents were tested in the two systems described above, but there was not a perfect correlation between the ability to bind to and inhibit calmodulin *in vitro* and the ability to inhibit TPA responses in intact cells. For example, 25 μM chlorpromazine caused similar effects on both EGF binding and choline incorporation as the same concentration of TFP. This phenothiozine antipsychotic agent has a lower affinity for calmodulin than TFP (5). A number of antipsychotic agents which are not based on the phenothiazine structure had little effect on the TPA responses. Thus haloperidol, which is only a weak calmodulin inhibitor, did not modify the TPA effects on either EGF binding or choline uptake when tested at 25 μM. In addition, penfluridol and pimozide which

are not phenothiazine derivatives, and are more effective calmodulin inhibitors than TFP (5), caused only minor inhibitions of the TPA responses at 25 μM concentrations. Differences in uptake and accessibility to target molecules may explain the disagreement between *in vitro* and *in vivo* potencies.

In preliminary experiments we have shown that the local anesthetic tetracaine (3 mM) also blocked the TPA inhibition of EGF binding. This result is interesting as it has recently been reported that this compound inhibits the induction of ornithine decarboxylase by TPA both *in vitro* and *in vivo* (22). There is one report that local anesthetics have anti-calmodulin activity (cited in ref. 23).

Some caution should be exercised in interpreting the effects of TFP on the two TPA responses. Although TFP and other antidepressant drugs bind strongly to calmodulin and inactivate it (5), it is not known whether this is the only biological effect of these drugs. As discussed by Cheung (6), TFP is hydrophobic and may partition non-specifically in membranes with, as yet, undetermined consequences. Never-the-less, the effect of TFP on the two TPA responses reported here was dramatic. As a minimum the data emphasises the need for a careful assessment of the role of Ca^{2+} in the biology of tumor promoters.

ACKNOWLEDGEMENTS.

Supported by grants from the Australian Research Grants Committee, the National Health and Medical Research Foundation and the Anti-Cancer Foundation of the Universities of South Australia. We are grateful to Mrs. Anne Rogers for excellent technical assistance.

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