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# 12-O-TETRADECANOYLPHORBOL-13-ACETATE STIMULATES RELEASE OF ARACHIDONIC ACID, PROSTAGLANDIN $E_2$ AND PROSTAGLANDIN $F_{2\alpha}$ FROM TPA NONPROLIFERATIVE VARIANTS OF 3T3 CELLS

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12-O-tetradecanoylphorbol-13-acetate is unable to stimulate DNA synthesis or cell division in the Swiss 3T3 variants 3T3-TNR-2 or 3T3-TNR-9. In contrast, 12-O-tetradecanoylphorbol-13-acetate stimulates release of arachidonic acid, prostaglandin E2 and prostaglandin F2α from the variant and parental cell lines. 12-O-tetradecanoylphorbol-13-acetate-stimulated release of these compounds is, therefore, not sufficient to stimulate mitogenesis. Although prostaglandin F2α is a potent mitogen for 3T3 cells, neither variant cell line responded to this compound.

The tumor promoter 12-O-tetradecanoylphorbol-13-acetate is a potent mitogen for Swiss 3T3 cells (1). One of the earliest events to occur in response to many mitogens is the stimulation of arachindonic acid and prostaglandin release (2) as a consequence of the activation of phospholipase $A_2$ (3). 3T3 cells produce prostaglandins $F_{2\alpha}$ and $E_2$ from released arachidonic acid. Because it begins very rapidly after mitogenic stimulation, release of arachidonic acid and subsequent prostaglandin production has been suggested as a potential causal step in the mitogenic response (4). One of the released products, $PGF_{2\alpha}$, is itself a potent mitogen for 3T3 cells (5).

We have selected two independently isolated variant cell lines, 3T3-TNR-2 and 3T3-TNR-9, which do not synthesize DNA or divide in response to TPA, but do respond to a variety of other mitogens (6). Despite a loss of the mitogenic

Abbreviations used: TPA, 12-O-tetradecanoylphorbol-13-acetate; $PGE_2$, prostaglandin E2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TLC, thin layer chromatography; FCS, fetal calf serum.

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response to TPA, these two variants have TPA receptors similar in number and affinity to the parent 3T3 cell line (7). In this study we investigated the release of arachidonic acid and prostaglandins $F_{2\alpha}$ and $E_2$ in response to TPA. Since arachidonic acid release and prostaglandin production was normal in 3T3-TNR-2 and 3T3-TNR-9, we then analyzed the mitogenic response of the TPA nonproliferative variants to prostaglandin $F_{2\alpha}$. Neither variant responded to prostaglandin $F_{2\alpha}$.

## METHODS

PGE2 and PGF2α were from Sigma. Phospholipid standards were the gift of Dr. James Mead. TPA was from Dr. Peter Borchert, University of Minnesota. [$^3$H]thymidine (20 Ci/mmol) and [$^3$H]arachidonic acid (78.2 Ci/mmol) were from New England Nuclear. Details of cell culture as well as variant isolation and characterization have been published previously (6). To measure incorporation of [$^3$H]thymidine cells were exposed to 0.1 μCi/ml for one hour and harvested as previously described (6). To label with arachidonic acid cells were plated in 35 mm dishes in Dulbecco's Modified Eagle's Medium containing 0.2% fetal calf serum (FCS). The following day cells were switched to medium containing 5% FCS and [$^3$H]arachidonic acid (4 μCi/ml). After three days the cells were washed with medium and used for experiments.

To measure arachidonic acid and prostaglandin release prelabeled cells were incubated with 0.6 ml conditioned medium either with or without TPA (100 ng/ml). At the appropriate times the medium was removed and the radioactivity of a 120 μl sample was determined. The remaining medium was acidified with 0.15 ml 0.1 N HCl and extracted two times with 0.75 ml ethyl acetate. The solvent was evaporated and the residue was redissolved in 20 μl ethyl acetate. The entire sample was then spotted on plastic backed silica gel TLC plates (J.T. Baker Co.). The samples were chromatographed in 2,6 dimethyl heptonone:glacial acetic acid:0.9% NaCl (80:40:6). Standards were visualized with iodine vapor. Spots were cut out, and radioactivity was determined.

## RESULTS

TPA-Stimulated Aarachidonic Acid Release: Growing Swiss 3T3 cells and the two TPA nonproliferative variants were labeled with tritiated arachidonic acid in order to produce labeled, density-arrested cells. To determine the amount of labeled arachidonic acid incorporated into the various cell lines, samples of each cell line were extracted in the presence of carrier lipid, and the radioactivity in the chloroform methanol (1:1) extract was measured, after washing with phosphate buffered saline. These values were $2.0 \times 10^5$ cpm/$10^5$ cells for 3T3, $2.1 \times 10^5$ cpm/$10^5$ cells for 3T3-TNR-2 and $1.6 \times 10^5$ cpm/$10^5$ cells for 3T3-TNR-9. Density-arrested cells were treated with TPA (100 ng/ml)

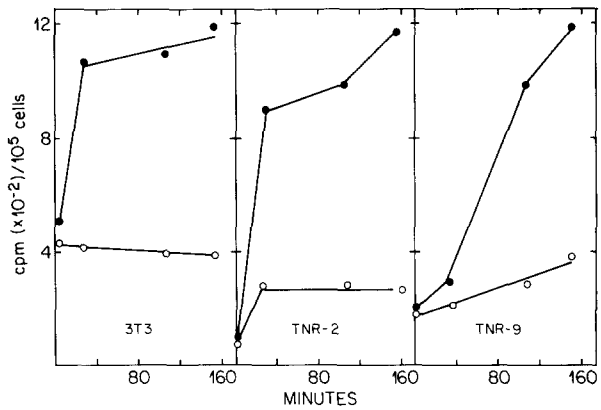


Figure 1. TPA stimulation of arachidonic acid release. Cells were prelabeled as described in Methods with tritiated arachidonic acid. Density-arrested prelabelled cells were treated with TPA (100 ng/ml) and arachidonic acid release into the medium was measured at the times indicated. All values are averages of duplicate determinations. ( ● ), TPA; ( O ), control.

and culture media were collected at 0, 30, 90, and 150 minutes. Lipids were extracted and arachidonic acid and its metabolites were separated by TLC. TPA stimulated equivalent levels of arachidonic acid release in the TPA responsive 3T3 cells and in the TPA nonproliferative variants 3T3-TNR-2 and 3T3-TNR-9 (Fig. 1). When parallel plates were tested for [$^3$H]thymidine incorporation, 3T3 cells responded to 10% serum and TPA (100 ng/ml), while the variants, as expected, responded only to serum (data not shown).

Table 1. Extracellular prostaglandin $F_{2\alpha}$ and $E_2$ release in response to TPA.

| Cells | $PGE_2$ (cpm/plate) | | $PGF_{2\alpha}$ (cpm/plate) | |
|---|---|---|---|---|
| | -TPA | TPA | -TPA | TPA |
| 3T3 | 1150 | 3553 | 108 | 296 |
| | 1256 | 3876 | 161 | 377 |
| 3T3-TNR-2 | 195 | 426 | 140 | 289 |
| | 256 | 497 | 184 | 344 |
| 3T3-TNR-9 | 172 | 295 | 189 | 586 |
| | 223 | 658 | 254 | 820 |

Radioactive PGF2α and PGE2 in the culture medium was measured 150 minutes after addition of TPA (100 ng/ml). There were $6.7 \times 10^5$, $4.1 \times 10^5$ and $5.6 \times 10^5$ cells per plate for 3T3, 3T3-TNR-2 and 3T3-TNR-9 cultures.

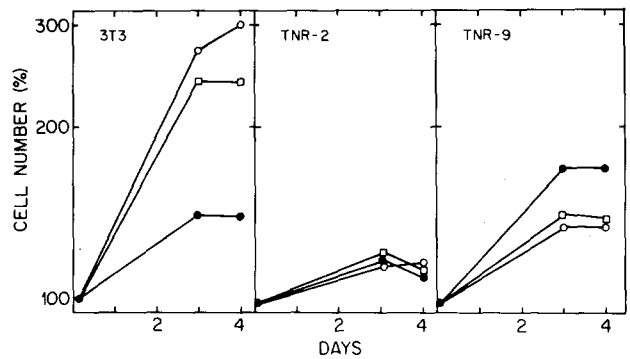


Figure 2. TPA and PGF2α stimulation of cell division. Density-arrested cultures of 3T3, 3T3-TNR-2 and 3T3-TNR-9 cells were treated with PGF2α (100 ng/ml) or TPA (100 ng/ml) and cell numbers were determined at the times indicated. Values are averages of duplicate determinations. ( ● ), control; ( O ), TPA; ( □ ) $PGF_{2\alpha}$.

Release of $PGE_2$ and $PGF_{2\alpha}$ in Response to TPA: The release of radioactive $PGF_{2\alpha}$ and $PGE_2$ was measured 150 minutes after addition of TPA (100 ng/ml) to density-arrested cultures of 3T3, 3T3-TNR-2, and 3T3-TNR-9 cells previously labeled with tritiated arachidonic acid (Table 1). There was a substantial difference in the basal levels of $PGE_2$ released by the wild type 3T3 cells and the variants. However, all three cell lines were stimulated to higher levels of $PGE_2$ in response to TPA. The basal levels of $PGF_{2\alpha}$ production were quite similar for wild-type 3T3 cells and the two variants. Stimulation by TPA of $PGF_{2\alpha}$ synthesis and release was comparable in 3T3 and the two TPA nonproliferative variants.

Mitogenic Response to $PGF_{2\alpha}$: PGF2α is a potent mitogen for 3T3 cells (5). Since TPA stimulated apparently normal $PGF_{2\alpha}$ production in the TPA nonproliferative variants, we assayed the mitogenic responsiveness of these cells to $PGF_{2\alpha}$. Density-arrested wild-type 3T3 cells were stimulated to divide in response to either TPA or $PGF_{2\alpha}$. In contrast, $PGF_{2\alpha}$ was unable to stimulate cell division in either TPA nonproliferative variant (Fig. 2). Elevation of serum to 10%, included as a positive control, stimulated at least a doubling in cell number in all three cell lines (data not shown).

## DISCUSSION

Induction of arachidonic acid release and/or prostaglandin synthesis in response to mitogens has been postulated to be causal in several mitogen

responses. Although $PGE_2$ synthesis is reported to be required for cell proliferation in mouse skin (4) type E prostaglandins are unable to stimulate cell division in 3T3 cells (5). We therefore attribute little significance to the fact that 3T3 cells produce greater basal and TPA-induced levels of $PGE_2$ than do the two TPA nonproliferative variants. Shier (3,8) has postulated that the increase in lysolethicin in the membrane resulting from the substantial release of arachidonic acid after phospholipase activation by various ligands could cause sufficient membrane perturbation to trigger regulatory biological responses, including mitogenesis. Our data suggest that such a generalized membrane change is not sufficient to generate a mitogenic response, since the release of arachidonic acid is equivalent in the two TPA nonproliferative variants to that observed in responsive 3T3 cells. Since these variants do respond to other mitogens that produce similar activation of phospholipase A2, this shared membrane perturbation cannot be sufficient to cause the mitogenic response.

As an alternate possibility, the stimulated production of prostaglandin $F_{2\alpha}$ synthesis might be causal in the TPA mitogenic response for 3T3 cells. This idea is attractive because $PGF_{2\alpha}$ is a potent mitogen for 3T3 cells (5). If the released prostaglandin $F2_{\alpha}$ mediated TPA-induced mitogenesis one would predict that TPA non-responsive variants would not respond to $PGF_{2\alpha}$. This is the result we observe. Shier (8) has, however, reported that serum stimulates both arachidonic acid release and production of "type F PGs" in 3T3 cells. If substantial amounts of $PGF_{2\alpha}$ are also released by serum in the TPA nonresponsive variants, then this compound cannot be the causal mediator of a common pathway for both serum and TPA-induced mitogenesis, since the TPA nonproliferative variants demonstrate a proliferative response to serum (6). Thus if $PGF_{2\alpha}$ is a mediator of both the serum and TPA mitogenesis pathways at least one other causal step, not shared with the serum stimulated pathway, must exist for TPA-induced mitogenesis. Moreover, pharmacologic experiments (9) have suggested that prostaglandin release is not causal in TPA-mediated mitogenesis of 3T3 cells.

The TPA receptors of 3T3-TNR-2 and 3T3-TNR-9 are equivalent in number and affinity to those of 3T3 cells (7). Our current data demonstrate that the TPA receptor-ligand interaction in these TPA nonproliferative variants is competent to transduce a biological signal, activation of phospholipase activity. Thus the block in the proliferation response to TPA in these variants must be distal to the receptor-ligand interaction and, perhaps, to initial receptor-mediated events. Our results contrast with those of Yamasaki et al. (10), who found that release of arachidonic acid, $PGE_2$ and $PGF_{2\alpha}$ were absent in an erythroleukemia line resistant to TPA-mediated inhibition of differentiation and TPA-induced adhesion. This phenotypic diversity in TPA non-responsive variants suggests that a number of causal biochemical parameters are involved in the biological responses to TPA.

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