Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 158-164

GENERATION OF 1,2-DIACYLGLYCEROL IN PLASMA MEMBRANES OF PHORBOL ESTER-TREATED MYOBLASTS

Robert I. Grove[1] and Steven D. Schimmel

Department of Biochemistry
University of South Florida College of Medicine
Tampa, Florida 33612

Received July 29,1981

SUMMARY: The potent tumor promoter 12-O-tetradecanoylphorbol 13-acetate causes a 2-fold increase in 1,2-diacylglycerol levels within 15-30 min in cultured chick embryo differentiated myoblasts. The weak tumor promoter 12-O-decanoylphorbol 13-acetate was 250 times less effective and the non-promoter 4-α-phorbol 12,13 didecanoate was ineffective at producing this response. During subcellular fractionation, the stimulated portion of the diacylglycerol was distributed similarly to the plasma membrane fraction. Evidence is presented that this diacylglycerol originates from pre-existing lipid rather than from de novo synthesis. Possible implications of these findings with regard to the inhibition of myoblast fusion by the tumor promoter are discussed.

Cultured cells treated with very low concentrations (10^{-8}-10^{-9}M) of the tumor promoter TPA[2] undergo a wide range of reversible alterations including alterations in cell morphology, growth control, plasma membrane properties, and differentiation (1,2). In the chick embryo myogenesis system, TPA reversibly inhibits the differentiation of precursor myogenic cells into mononucleated myoblasts (3) as well as the fusion of differentiated myoblasts to form multinucleated myotubes (3,4). A variety of other plasma membrane functions are altered in TPA-treated differentiated myoblasts, including Ca^{++} and hexose transport (5,6) and cyclic nucleotide metabolism (7). Since the lipid components of plasma membranes are critically important for the maintenance of normal membrane structure and function and since several TPA-dependent alterations in lipid metabolism have been reported to occur in other cell types

[1] Present address, to which correspondence should be sent:
NIEHS, RM 418 BLD 4;
NIH P. O. Box 12233;
Research Triangle Park, NC 27709

[2] Abbreviation: TPA, 12-O-tetradecanoylphorbol 13-acetate

0006-291X/81/170158-07$01.00/0

(1,8-11), an investigation of the effect of TPA on myoblast lipid metabolism was undertaken.

MATERIALS AND METHODS

Phorbol esters, lipid standards, and α-bungarotoxin were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Glycerol (9.5 Ci/mol), [^{14}C]glycerol (152 mCi/mmol) and $^{125}I^{-}$ were purchased from New England Nuclear (Boston, MA). [^{125}I]α-Bungarotoxin was prepared as described previously (12). Myoblast cell cultures were prepared from breast muscle of 12-day white leghorn chick embryos essentially as described previously (12), except that 2.5 mg/ml collagenase (Worthington Type III) was used in place of trypsin. Cells (1.5×10^{6}/60 mm dish) were grown in Ca^{++}-free DMEM containing 10% fetal bovine serum (treated with Chelex-100 resin) and 1% 11-day chick embryo extract. The final concentration of Ca^{++} in the medium was adjusted to 0.1 mM, which permits the normal differentiation of mononucleated myoblasts but prevents myotube formation (13). After 30-40 hours, the cells were fed with fresh medium containing 10^{-5}M cytosine arabinoside to kill any proliferating cells. Three-day cultures were used for all experiments. Variation in protein content per dish was <7%. Subcellular fractionation was performed as described previously (12). Protein assays were by the method of Lowry (14).

Lipid analysis - Cells or subcellular fractions were solubilized in 0.2% sodium dodecyl sulfate. Lipids were extracted by the method of Bligh and Dyer (15). Neutral lipids were separated on Silica Gel-60 precoated thin-layer plates (E. Merck Reagents) developed with benzene/ethyl acetate (7:3) (15). Lipid standards were located with iodine vapor and the corresponding radioactive diacylglycerol region was counted in a gel dispersion containing 1.5 ml water and 3 ml ACS scintillation cocktail (Amersham).

RESULTS

As shown in Figure 1, when differentiated myoblasts were labeled with [^{3}H]glycerol for 24 h and then treated with TPA, a 2-fold increase in labeled diacylglycerol levels occurred within 30 min. The levels of label in triacylglycerol and phospholipids remained unchanged (not shown). In both control and TPA-treated cells, >75% of labeled glycerol remained in the glycerol moiety of the diacylglycerol, as determined by saponification. In order to investigate the specificity of this response, the ability of other phorbol esters to elicit increased diacylglycerol was determined. As shown in Figure 2, the weak tumor promoter, 12-O-decanoylphorbol 13-acetate, also stimulated diacylglycerol labeling, but at approximately 250 times the concentration of TPA required for half-maximal stimulation. The non-promoting analogue 4-α-phorbol 12,13-didecanoate, which presumably has amphiphilic properties similar to TPA, had no diacylglycerol-stimulating activity, suggesting that the effect of TPA was not due to non-specific membrane perturbations.

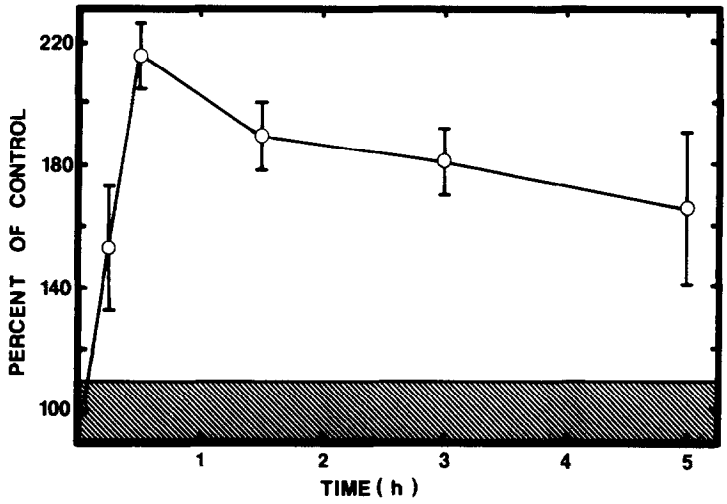

Fig. 1: Effect of TPA on 1,2-diacylglycerol levels. At 44 h post-plating, 5 μCi/ml [^{3}H]glycerol was added to myoblast cultures. TPA (20 ng/ml), dissolved in acetone, was added after an additional 24 h. Acetone alone (0.2% final concentration) was added to control cultures. At the indicated times, the cells were solubilized and the amount of labeled 1,2-diacylglycerol was determined as described in Methods. The data points represent the means ± S.D. of triplicate 60 mm dishes, and the control value (780 ± 80 cpm) was obtained from five determinations.

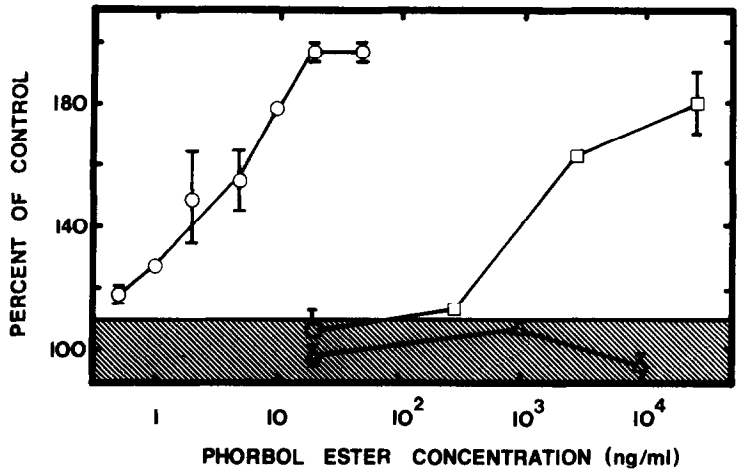

Fig. 2: Phorbol ester specificity of the diacylglycerol response. Myoblasts grown in 60 mm culture dishes were labeled for 24 h (44 h to 68 h post-plating) with 10 μCi/ml [^{3}H]glycerol, after which the indicated amounts of TPA (○), 12-O-decanoylphorbol 13-acetate (□) or 4-α-phorbol 12,13-didecanoate (△) were added for 45 min. Myoblasts were solubilized and the diacylglycerol was extracted, chromatographed and counted as described in Methods. Data points are the means ± 1/2 the range for n = 2, except for the point at 1 μg/ml 4-α-phorbol 12,13-didecanoate, which is from a single determination. Values for TPA are from 2 separate experiments. Control values were 700 ± 70 cpm diacylglycerol per dish.

The TPA-dependent stimulation of diacylglycerol labeling could have arisen from a stimulation of de novo synthesis or from a stimulation of the turnover of existing glycerolipids to give diacylglycerol. To distinguish between these possibilities, the effects of TPA on diacylglycerol labeling were compared in myoblasts labeled with glycerol during or prior to TPA treatment. As shown in Table I, cells labeled for 40 h prior to TPA addition were stimulated by 61%; those labeled only 4 h prior to TPA addition and 2 h during TPA treatment were stimulated 22%; and those pulse-labeled for 15 min at 75 min following TPA addition were not significantly stimulated. These results suggest that TPA stimulates the generation of diacylglycerol from pre-existing lipid and has no effect on de novo diacylglycerol synthesis.

To determine the possible subcellular location(s) of the TPA-stimulated generation of diacylglycerol, myoblasts labeled with radioactive glycerol were treated with TPA and then fractionated using differential and sucrose

TABLE I

EFFECT OF GLYCEROL LABELING PERIOD ON TPA-DEPENDENT DIACYLGLYCEROL RESPONSE

Experiment Number	Labeling Period hours (interval post-plating)		Diacylglycerol cpm/dish		Percent Stimulation
			control	TPA	
1[a]	40	(24-64)	6200	10000	61
2[b]	6	(66-72)	12000	14600	22
3[c]	0.25	(71:15-71:30)	1150 ± 110	1200 ± 50	4

Myoblasts were labeled with [^{14}C]- or [^{3}H]glycerol for different lengths of time at the indicated intervals after plating. TPA (20 ng/ml) was added at 70 h post-plating. At 72 h (71.5 h for Experiment 3) cells were solubilized and the diacylglycerol was extracted and quantitated as described in Methods.

[a] Cells in 100 mm dishes were labeled with 5 μCi/ml [^{14}C]glycerol (152 mCi/mmol). At 64 h post-plating, the medium was replaced with unlabeled conditioned medium obtained from parallel unlabeled cultures.

[b] Cells in 100 mm dishes were labeled with 20 μCi/ml [^{3}H]glycerol (152 mCi/mmol).

[c] Cells in 60 mm dishes were labeled with 100 μCi/ml [^{3}H]glycerol (9.5 Ci/mmol). Values are the mean ± S.D. of triplicate cultures.

density gradient centrifugation (12). When the relative specific activities of the TPA-stimulated diacylglycerol and a plasma membrane marker ([^{125}I]α-bungarotoxin bound to surface acetylcholine receptors, ref. 12) were compared (Figure 3), both were clearly enriched in the 13-27% sucrose interface fraction, the diacylglycerol to a somewhat greater extent than the α-bungarotoxin. These results suggest that the TPA-stimulated diacylglycerol is located either in the plasma membrane or in a fraction similar in density and size to the plasma membrane fraction.

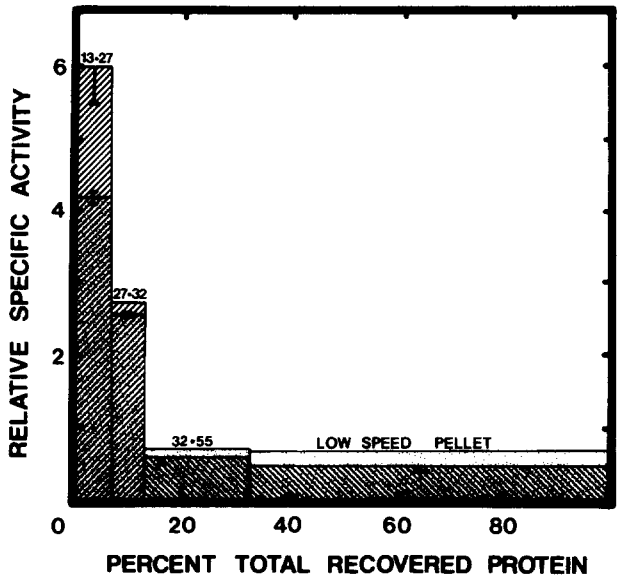

Fig. 3: Myoblasts grown in two 150 mm diam. culture dishes were labeled for 24 h (44 h to 68 h post-plating) with 5 μCi/ml [^{3}H]glycerol. TPA (20 ng/ml) was added to one dish 90 min before harvest. Ten min before harvest, cells were incubated with 10^{7} cpm (Experiment I) or 2 X 10^{7} cpm (Experiment II) of [^{125}I]α-bungarotoxin. The cells were then harvested and fractionated as described previously (12). Diacylglycerol from each fraction was extracted and quantitated as described in Methods. Experiments I & II were done on separate days. Total recoveries of diacylglycerol in Experiment I were 75% and 84%, respectively, for TPA-treated and control cells. The corresponding recoveries in Experiment II were 103% and 90%. Values for total TPA-stimulated diacylglycerol were 4200 cpm (Experiment I) and 6350 cpm (Experiment II). Values for total ^{125}I cpm were 8400 (control) and 7250 (TPA) for Experiment I and 15400 (control) and 19300 (TPA) for Experiment II. Relative specific activity was calculated by dividing the percent total TPA-stimulated diacylglycerol or percent total α-bungarotoxin in each fraction by the percent of total recovered protein in that fraction (12). Stippled bars represent α-bungarotoxin; cross-hatched bars represent TPA-stimulated diacylglycerol. The height of each bar represents the average of Experiments I and II and the error bars indicate the range. The area of each bar is proportional to the radioactivity of α-bungarotoxin or diacylglycerol found in that fraction.

DISCUSSION

A wide variety of alterations in lipid metabolism have been reported to occur in TPA-treated non-myogenic cells. These include increased deacylation of cellular lipids and synthesis of prostaglandins (10,11), increased incorporation of choline (1,8,9), and increased incorporation of ^{32}P, inositol and acetate (1). It is possible that many of the alterations in lipid metabolism cited above could be secondary to increased diacylglycerol generation. For example, Sleight and Kent (17) demonstrated that the generation of diacylglycerol in myoblast plasma membrane by exogenous phospholipase C caused a 3 to 5-fold stimulation of choline incorporation into phosphatidylcholine. Perhaps a similar mechanism is responsible for the increased choline incorporation observed in TPA-treated cells (1,8,9).

The generation of diacylglycerol in the plasma membrane of myoblasts may have wide-ranging effects on membrane function. Nameroff et. al.(18) and Kent (19) demonstrated that the generation of diacylglycerol in the myoblast plasma membrane using exogenous phospholipase C resulted in the inhibition of myoblast fusion. A possible mechanism for this inhibition and that caused by TPA (3,4) could involve the diffusion of enzyme-generated diacylglycerol from the outer leaflet of the membrane to the inner leaflet. According to the bilayer couple hypothesis (20), the net transfer of mass to the inner membrane leaflet would promote invagination and inhibit evagination of the plasma membrane. This could inhibit cell-cell fusion by 1) increasing the distance between fusion sites in the apposing membranes, and 2) preventing microprocess formation thought to be required for fusion (21). Similar reasoning would lead to the expection that TPA should promote secretion-associated fusion of intracellular vesicles with the plasma membrane on the cytosolic side. Consistent with this prediction are the reports that TPA stimulated secretion of lysosomal enzymes (22,23), insulin (24) and histamine (25). Further support comes from the finding that mast cells exhibit a 2-fold increase in levels of diacylglycerol within 1 min when stimulated with the histamine-releasing agent 48/80 (26). Thus it is possible

that the production of diacylglycerol in the plasma membrane of TPA-treated myoblasts could explain how TPA inhibits myoblast fusion.

ACKNOWLEDGEMENTS

This work was supported in part by grant NS12558 from the National Institutes of Health. The authors wish to acknowledge the excellent technical assistance of Ronald Kirkland.

REFERENCES

1. Blumberg, P.M. (1980) CRC Critical Reviews-Toxicology 8, 153-197.
2. Weinstein, I.B., Lea, L., Fisher, P.B., Mufson, A., and Yamasaki, H. (1979) J. Supramol. Struct. 12, 195-208.
3. Cohen, R., Pacifici, M., Rubinstein, N., Biehl, J., and Holtzer, H. (1977) Nature 266, 538-540.
4. Keldie, V.C., Grove, R.I., and Schimmel, S.D. (1977) Abstr. 29th Meeting Southeast Regional American Chemical Society.
5. Schimmel, S.D., and Hallam, T. (1980) Biochem. Biophys. Res. Commun. 92, 624-630.
6. Schimmel, S.D., and Keldie, V.C. (1978) Fed. Proc. 37, 1817.
7. Grotendorst, G.R., and Schimmel, S.D. (1980) Biochem. Biophys. Res. Commun 93, 301-307.
8. Suss, R., Kinzel, V., and Kreibich, G. (1971) Experientia 27, 46-47.
9. Cabot, M.C., Welsh, C.J., Callahan, M.F., and Huberman, E. (1980) Cancer Res. 40, 3674-3679.
10. Levine, L., and Hassid, A. (1977) Biochem. Biophys. Res. Commun. 79, 477-484.
11. Mufson, R.A., De Feo, D., and Weinstein, I.B. (1979) Molec. Pharm. 16, 569-578.
12. Schimmel, S.D., Kent, C., Bischoff, R., and Vagelos, P.R. (1973) Proc. Natl. Acad. Sci. USA 70, 3195-3199.
13. Paterson, B., and Strohman, R.C. (1972) Develop. Biol. 29, 113-138.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
16. Keller, R.K., Adair, W.L., and Ness, G.C. (1979) J. Biol. Chem. 254, 9966-9969.
17. Sleight, R., and Kent, C.J. (1980) J. Biol. Chem. 255, 10644-10650.
18. Nameroff, M., Trotter, J.A., Keller, J.M., and Munar, E. (1973) J. Cell Biol. 58, 107-118.
19. Kent, C. (1979) Proc. Natl. Acad. Sci. USA 76, 4474-4478.
20. Sheetz, M.P., and Singer, S.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4457-4461.
21. Poste, G., and Allison, A.C. (1973) Biochim. Biophys. Acta. 300, 421-465.
22. Goldstein, I.M., Hoffstein, S.T., and Weissman, G.J. (1975) J. Cell Biol. 66, 647-652.
23. White, J.G., and Estenson, R.D. (1974) Am. J. Pathol. 75, 45-60.
24. Virji, M.A.G., Steffes, M.W., and Estenson, R.D. (1978) Endocrinology 102, 706-711.
25. Schleimer, R.P., Gillespie, E., and Lichtenstein, L.M. (1981) J. Immunol. 126, 570-574.
26. Kennerly, D.A., Sullivan, T.J., Sylvester, P., and Parker, C.W. (1979) J. Exp. Med. 150, 1039-1044.