Journal of Cellular Biochemistry 37:339–345 (1988)

Effect of Transforming Growth Factor-α on Inositol Phospholipid Metabolism in Human Epidermoid Carcinoma Cells

Miwako Kato, Tadaomi Takenawa, and Daniel R. Twardzik

Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173, Japan (M.K., T.T.); Oncogen, 3005 First Avenue, Seattle, Washington 98121 (D.R.T.)

Transforming growth factor-α (TGF-α) stimulates (in a dose-dependent manner) the incorporation of [^{32}P]Pi into phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2), and phosphatidic acid (PA) in the human epidermoid carcinoma cell line (A431). The effect of TGF-α on the incorporation was found to be similar to that of EGF. On the other hand, a striking difference in the activation of diacylglycerol (DG) kinase activity was seen between TGF-α and EGF. At least 100 times more TGF-α was required to achieve maximal stimulation of DG kinase activity relative to EGF. These results suggest that the activation of DG kinase by TGF-α may involve a mechanism independent from or subsequent to activation of the EGF receptor.

Key words: diacylglycerol kinase activity, signal transduction, epidermal growth factor

Transforming growth factor-α (TGF-α) is a potent mitogenic 50-amino-acid peptide originally isolated from media conditioned by retroviral-transformed rodent cells [1–3]. Rat TGF-α has been sequenced [4], cloned [5], and chemically synthesized [6]. Both rat and human TGF-α [7] share structured homology with epidermal growth factor (EGF); the six cysteines which are known to be important for secondary structure of EGF are found in homologous positions in TGF-α [8]. TGF-α binds to and stimulates tyrosine specific kinase activity in EGF membrane receptors with kinetics similar to EGF [9] and is thus equipotent to TGF in numerous biological systems [current review, 10].

Recently it has been found that some growth factors such as platelet-derived growth factor, bombesin, and bradykinin stimulate inositol phospholipid metabolism [11–13]. It has been suggested that these growth factors induce cell proliferation through the enhancement of inositol phospholipid metabolism; the primary event has been thought

Abbreviations used: TGF-α, transforming growth factor-α; EGF, epidermal growth factor; PI, phosphatidylinositol; PI(4), phosphatidylinositol 4-phosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; DG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; DOC, sodium deoxycholate.

Received June 9, 1987; accepted November 11, 1987.

to be breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3) [14–16]. DG is known to act as an activator for protein kinase C [16] and IP_3 acts as a second messenger for Ca^{2+} release from intracellular stores [14,15]. The human epidermoid carcinoma cell line (A431) has an unusually high number of EGF receptors [17,18] and in these cells EGF stimulates inositol phospholipid metabolism [19]. Such an effect, however, has not been shown in 3T3 fibroblasts [20]. We have recently shown that EGF stimulates DG kinase activity in isolated plasma membranes from A431 cells [21]. In this study we describe the effects of TGF-α and EGF on inositol phospholipid metabolism and DG kinase activity.

MATERIALS AND METHODS

Materials

Mouse EGF was purchased from Collaborative Research, Inc., Waltham, MA. TGF-α was purified from serum-free media conditioned by Snyder Theilen Feline Sarcoma Virus–transformed Fisher rat embryo cell as previously described [2]. The concentration of TGF-α is expressed as EGF receptor equivalents and was determined in EGF receptor competition assays on formaldehyde-fixed A431 cells by using mouse submaxillary gland EGF as a reference standard. Purity was determined by amino terminal sequence analysis as previously described [4]. Dioleoylglycerol, phosphatidylinositol, and phosphatidic acid were from Sedary Res. Lab., Ontario, CA; phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were purified from bovine spinal cord [22]. [^{32}P]ATP and [^{32}P]orthophosphoric acid (carrier free) were from New England Nuclear. A431 cells were grown in RPMI medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) buffer, pH 7.4, 100 units/ml of penicillin, streptomycin, and 10% fetal calf serum.

Inositol Phospholipid Metabolism

Cells were washed three times with phosphate-free DMEM and then scraped with a rubber policeman. The cells were washed three times with the fresh medium containing 10% fetal calf serum, which was dialyzed against phosphate-free DMEM and then aliquots of the suspended cells ($1–2 \times 10^6$ cells/0.5 ml) were labeled with [^{32}P]Pi(0.1mCi/ml) in the presence of EGF or TGF-α for the indicated time. The reaction was stopped by adding 3 ml of chloroform/methanol (1:1, v/v). Extracts were partitioned into two phases after an addition of 400 μl of 1 N HCl. The lower phase was washed with synthetic upper phase and evaporated to dryness under a stream of nitrogen. After PI(4)P and PI(4,5)P_2 were added as carrier, polyphosphoinositides were separated with thin-layer chromatography as described before [23]. Each lipid was visualized by exposure to iodine vapor and also by autoradiography. PI(4)P and PI(4,5)P_2 fractions were scraped off and the radioactivity was measured in a liquid scintillation counter.

Preparation of Plasma Membranes

A431 cells were grown to 80–90% confluency, and then shed plasma membrane vesicles were isolated by a modification of the method of Cohen et al. [24], in which the hypotonic buffer and the vesiculation buffer contained 2 mM EGTA and 100 μM phenylmethylsulfonyl fluoride.

Assay of DG Kinase

DG kinase activity was measured by the method of Kanoh et al. [25]. The standard reaction mixture consisted of 50 mM Tris/HCl buffer (pH, 7.4), 10 mM $MgCl_2$, 0.5 mM mercaptoethanol, 20 mM NaF, 1 mM sodium deoxycholate, 1 mM [γ-^{32}P]ATP(2-10 μCi), and 10 mM dioleoylglycerol in a total volume of 50 μl. Reaction was started by the addition of plasma membrane vesicles (5–15 μg of protein) at 30°C and then stopped by adding 2 ml of chloroform/methanol (2:1, v/v) and 400 μl of 1 M KCl. The mixture was vortexed and separated into two phases by centrifugation at 1,000g for 5 min. After the lower phase was washed with synthetic upper layer, 5 μg of PA was added to the lower phase. The solution was evaporated to dryness and spotted on thin-layer plates (precoated silica gel G plate, Merck). The plate was developed by chloroform/methanol/water (65:25:4,v/v/v). PA fraction was scraped off and the radioactivity was counted.

RESULTS

The effect of TGF-α on the incorporation of [^{32}P]Pi into phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2), and phosphatidic acid (PA) into A431 cells was compared to that of EGF. As shown in Figure 1, treatment of A431 cells with equimolar amounts (10 ng/ml) of either TGF-α or EGF stimulated the incorporation of [^{32}P]Pi into PIP, PIP_2, PI, and PA. Maximal stimulation in [^{32}P]Pi incorporation (approximately twofold) was observed in

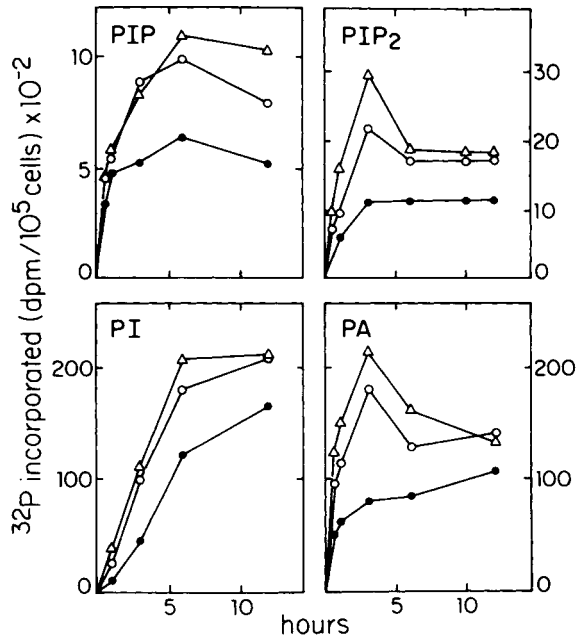

Fig. 1. Time course of [^{32}P]Pi incorporation into inositol phospholipids and phosphatidic acid. A431 cells were washed three times with phosphate-free DMEM, scraped, and resuspended (1×10^6 cells/0.5 ml) in phosphate-free medium containing 10% dialyzed fetal calf serum. Cells were then incubated with [^{32}P]P_i (50μ C_i/tube) for the indicated time at 37°C in the presence of 10 ng/ml of TGF-α (●); or EGF (△); or without any additions (○).

6 hr for PIP; both PIP_2 and PA show maximal enhancement at 2.5 hr with a reduction in extent seen at 12 hr. In contrast, the kinetic for both TGF-α- and EGF-stimulated incorporation of [^{32}P]Pi into PI appeared more extended (12 hr) with little degradation of measured product. No differences were seen between TGF-α and EGF; however, TGF-α was not as effective as EGF in stimulating the incorporation of [^{32}P]Pi into phosphatidylcholine or phosphatidylethanolamine (data not shown). The stimulation of incorporation of [^{32}P]Pi into PI, PA, PIP, and PIP_2 was dependent on the concentration of TGF-α used. A dose-response curve is shown in Figure 2. In the presence of as low as 0.1 ng/ml of TGF-α a stimulation was observed. Maximal incorporation of [^{32}P]Pi into PI, PA, PIP, and PIP_2 was observed at 10 ng/ml. Higher doses were not tested.

We have previously shown [21] that EGF stimulates DG kinase activity in isolated plasma membrane vesicles prepared from A431 cells. As shown in Figure 3, TGF-α also stimulated, relative to untreated (no growth factor) controls, DG kinase activity approximately twofold during the 20-min incubation period. EGF, however, was more potent in stimulated DG kinase activity than was TGF-α (Fig. 4). Whereas a maximal stimulation of enzyme activity was observed in A431 plasma membranes at approximately 10 ng/ml of EGF, it required much higher concentrations, well over two orders of magnitude, of TGF-α to achieve a comparable result. A similar dose-dependent activation of DG kinase activity by EGF was also observed in partially purified enzyme preparation derived from the soluble nonmembrane fraction of A431 cells (T. Takenawa, personal communication). This effect could not be attributed to the preferential degradation of TGF-α in the preparation as similar amounts of both EGF and TGF-α could be recovered from these preparations postincubation.

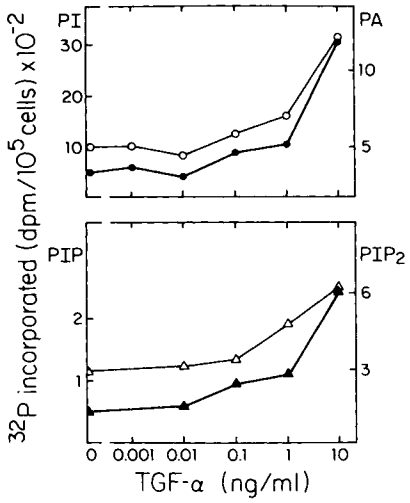

Fig. 2. Dose-response curve of TGF-α on the incorporation of [^{32}P]Pi into PI, PIP, PIP_2, and PA. A431 cells ($5-10^6$ cells/0.5 ml) were incubated with 10 μCi of [^{32}P]Pi in the presence of various concentrations of TGF-α for 6 hr. PI (○), PA (●), PIP_2 (△), PIP (▲).

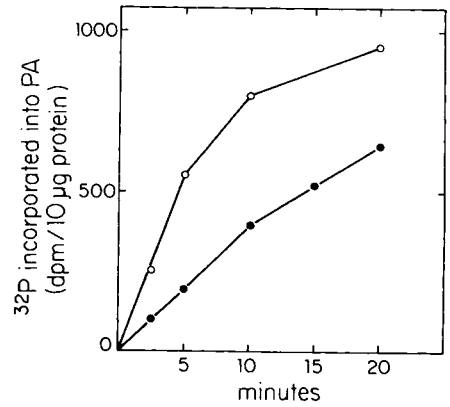

Fig. 3. Time course of PA formation by DG kinase. Plasma membrane vesicles from A431 cells were incubated at 30°C for the indicated time in the presence (○) or absence (●) of 1 μM of TGF-α, as described in Materials and Methods.

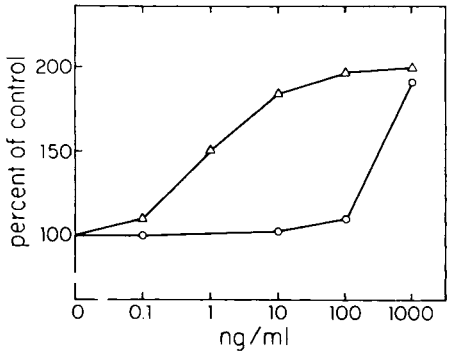

Fig. 4. Dose-response curves for TGF-α-stimulated PA formation. DG kinase activity was measured at 30°C for 10 min with 1 mM [^{32}P] ATP and 10 mM DG in the presence of various concentrations of TGF-α (○) or EGF (△).

DISCUSSION

TGF-α shares structural homology with EGF most notably in the conservation of the alignment of all six cysteine residues. Thus the resultant similarity in the disulfide-bond-mediated secondary structure of both molecules is thought to result in the observed equivalent functionality [10]. In that regard, both TGF-α and EGF bind to the 180-kd membrane receptor for EGF with similar kinetics and stoichiometry and stimulate tyrosine-specific autophosphorylation of the receptor [9,18]. As a result, many studies in disparate biological systems have demonstrated indistinguishable effects for both TGF-α and EGF [26]. For example, both TGF-α and EGF have been shown to stimulate anchorage-independent cell growth of rodent fibroblasts in the presence of TGF-β [27]. Likewise, both molecules attenuate the acquisition of aromatase activity by granulosa cells [28], induce a similar early cell-ruffling response [29], and induce accelerated tooth eruption in newborn mice [30]. Likewise, in this study we have shown that TGF-α exhibited effects similar to EGF in enhancing inositol phospholipid metabolism. Treatment of EGF-receptor-rich A431 cells with nM amounts of TGF-α stimulated PI, PIP, PIP_2, and PA metabolism. These results are similar to that previously reported by Sawyer and Cohen for EGF stimulation of phosphatidylinositol turnover in A431 cells [19]. In

their system, however, they observed a 5–10-fold stimulation by EGF in the incorporation of ^{32}[P]Pi into product whereas we consistently noted only a 2–3-fold effect. More importantly, however, was that we noted similar results, quantitatively, for both TGF-α and EGF in a homologous system. It has been suggested that the EGF-stimulated calcium influx signals stimulation of phosphatidylinositol turnover [19,31]. TGF-α also stimulates calcium transport (C. Werner, personal communication); thus some of the diverse biological effects induced similarly by TGF-α and EGF could involve a similar mechanism(s) of action. The most striking effect observed in this study was the major difference between TGF-α and EGF in the activation of DG kinase. Dose-response experiments showed that TGF-α was 100–1,000 times less potent than EGF in this regard though maximal activation was almost similar. In this regard, experiments were conducted (data not shown) which ruled out the preferential degradation of TGF-α in DG kinase assays containing plasma membrane vesicles. These results suggest that the mechanism for the activation of DG kinase is different from and may be mediated through a pathway independent of binding to and stimulating the EGF receptor. Degradation of PIP_2 to DG and inositol 4,5-bisphosphate by phospholipase C has been proposed to be a primary event in stimulus-response coupling in a variety of different tissues [review, 32]. Usually, newly formed DG is converted very rapidly to PA by DG kinase. Since DG kinase is responsible for the half-life of DG, it plays important roles in regulation of protein kinase C activity through competition for DG. The treatment of EGF or TGF-α on A431 cells enhances the breakdown of PIP_2, resulting in protein kinase C activation. However, if EGF or TGF-α stimulates DG kinase activity by a different mechanism, it is presumed that protein kinase C activation is suppressed. In the experiments we describe, however, the activation effect of TGF-α on DG kinase is not seen at physiological concentrations of TGF-α. This result possibly suggests that suppression of protein kinase C does not occur under physiological conditions with TGF-α, and thus, a different mechanism may be proposed. Again, although TGF-α and EGF have been generalized as producing similar effects, there are some exceptions. TGF-α is 3–10-fold more potent than EGF in inducing calcium release in the rat calvaria [33] and 10–100-fold more potent than EGF in stimulating proliferation of *osteoclast* precursor cells [34]. Likewise, TGF-α induces neovascularization in a system where EGF does so poorly [35]. Therefore, although TGF-α and EGF bind to stimulate equivalent autophosphorylation of the same receptor, proposing a similar, unified mechanism of action is inconsistent with the diverse effect seen in some target cells and tissues. Perhaps the differential effect (as shown in this study) of TGF-α and EGF on DG kinase activation provides a clue that alternate pathways subsequent to cell membrane receptor-ligand interactions are also determinants of a particular target tissue responsiveness to a given growth factor.

ACKNOWLEDGMENTS

Thanks to Jane Ranchalis for helpful suggestions and to Phyllis Yoshida and Cyndy Becker for assistance in preparation of the manuscript.

REFERENCES

1. DeLarco JE, Todaro GJ: Proc Natl Acad Sci USA 75:4001, 1978.
2. Twardzik DR, Todaro GJ, Marquardt H, Reynolds FH, Jr, Stephenson JR: Science 216:894, 1982.

3. Ozanne B, Fulton RJ, Kaplan PL: J Cell Physiol 105:163–180, 1980.
4. Marquardt H, Hunkapillar MW, Hood LE, Todaro GJ: Science 233:1079, 1984.
5. Lee DC, Rose TM, Webb NR, Todaro GJ: Nature 313:489, 1985.
6. Tam JP, Marquardt H, Roseberger D, Wong TW, Todaro GJ: Nature 309:376, 1984.
7. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV: Cell 38:287, 1984.
8. Marquardt H, Hunkapiller MW, Hood LE, Twardzik DR, DeLarco JE, Stephenson JA, Todaro GJ: Proc Natl Acad Sci USA 80:4684, 1983.
9. Reynolds FH, Todaro GJ, Fryling C, Stephenson JR: Nature 292:259, 1984.
10. Twardzik DR, Ranchalis JR: UCLA Symposium on Bone and Cartilage, A Leis, 1987.
11. Brown KD, Blag J, Irvine RF, Heslop JP, Berridge MJ: Biochem Biophys Res Commun 123:377, 1984.
12. Moolenaar WH, Tertoolen LGL, Laat SW: J Biol Chem 259:8066, 1984.
13. Morris JDH, Metcalfe JC, Smith GA, Hesketh TR, Taylor MV: FEBS Lett 169:189, 1984.
14. Berridge MJ: Biochem J 220:345, 1984.
15. Berridge MJ, Irvine RJ: Nature 312:315, 1984.
16. Nishizuka Y: Nature 308:693, 1984.
17. Fabricant RN, Delavco JE, Todaro GJ: Proc Natl Acad Sci USA 74:565, 1977.
18. Massague J: Prog Med Virol 32:142, 1985.
19. Sawyer ST, Cohen S: Biochemistry 20:6280, 1981.
20. MacPhee CM, Drummond AH, Otto AM, Jiminez de Asua L: J Cell Physiol 119:35, 1984.
21. Kato M, Homma Y, Nagai Y, Takenawa T: Biochem Biophys Res Commun 129:375, 1985.
22. Palmer FB St C: J Lipid Res 22:1296, 1981.
23. Kato M, Kawai S, Takenawa T: J Biol Chem 262:5696, 1987.
24. Cohen S, Ushiro H, Stoscheck L, Chinkers M: J Biol Chem 10:1523, 1982.
25. Kanoh H, Kondoh H, Ono T: J Biol Chem 258:1767, 1983.
26. Derynck R: J Cell Biochem 32:293, 1986.
27. Anzano MA, Roberts AB, Smith JM, Sporn MB, Delato JE: Proc Natl Acad Sci USA 80:6264, 1983.
28. Adashi EY, Resnick CE, Twardzik DR: J Cell Biochem 33:1, 1987.
29. Myrdal S, Twardzik DR, Auersperg N: J Cell Biol 102:1230, 1986.
30. Smith JM, Sporn MB, Roberts AB, Derynck R, Winkler ME, Gregory H: Nature 315:515, 1985.
31. Michell RH: Biochim Biophys Acta 415:81, 1975.
32. Berridge MJ, Irvine RF: Nature 312:315, 1984.
33. Ibbotson KJ, Twardzik DR, D'Souza SM, Hargreaves WR, Mundy GB: Science 228:1007, 1985.
34. Ibbotson KJ, Harrod J, Gowen M, D'Souza S, Winkler ME, Derynck R, Munday GR: Proc Natl Acad Sci USA 83:3278, 1986.
35. Schrieber AB, Winkler ME, Derynck R: Science 232:1250, 1986.