Vol. 173, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1990 Pages 208-211

INHIBITION OF EGF-INDUCED PHOSPHOLIPASE C ACTIVATION IN A431 CELLS BY ERBSTATIN, A TYROSINE KINASE INHIBITOR

Masaya IMOTO, Naomi SHIMURA, Hideaki UI,
and Kazuo UMEZAWA

Department of Applied Chemistry, Faculty of Science and Technology,
Keio University
3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223, Japan

Received October 18, 1990

Summary: Erbstatin, a tyrosine kinase inhibitor, inhibited epidermal growth factor (EGF)-induced inositol phosphate production in cultured A431 cells. However, it did not inhibit ATP-induced inositol phosphate production. Cytosolic but not membrane-associated phospholipase C was activated by EGF, and erbstatin inhibited enhancement of the phospholipase C activity in EGF-treated cells. Thus, tyrosine kinase of A431 cells is suggested to be functionally involved in phospholipase C activation.

Many oncogene products and growth factor receptors have tyrosine kinase activity (1). Therefore, tyrosine phosphorylation of proteins may be implicated both in oncogenic transformation and in mitogenic signal transduction. However, the details of the signal transduction process at the molecular level from tyrosine kinase activation to mitogenesis or transformation are not yet clear. Especially, the intracellular target substrate of the tyrosine kinase is unknown.

Phospholipase C (PLC) is the key enzyme of phosphatidylinositol (PtdIns) turnover, and the activation of this enzyme causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, thereby forming two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, which activate protein kinase C and Ca^{2+} mobilization, respectively (2,3). Recently, "cross-talk" between tyrosine kinase and PtdIns turnover was suggested in several publications. Activation of receptors having tyrosine kinase by PDGF and EGF caused the stimulation of PtdIns turnover in cell culture (4,5); and moreover, phospholipase C-γ, an isozyme of phospholipase C, was phosphorylated at tyrosine residues in the growth factor-treated cells and could be directly tyrosine-phosphorylated by purified EGF receptor (6-8). Therefore, phospholipase C-γ is considered to be a possible substrate for tyrosine kinase, and one of the roles of tyrosine kinase in the signal transduction system may be the activation of PtdIns turnover. However, whether tyrosine phosphorylation of phospholipase C functionally modulates the activity of this enzyme in cell cultrue is unclear. Therefore, we have studied the functional role of tyrosine kinase by using erbstatin, a low molecular weight specific inhibitor of this enzyme (9).

Materials and Methods

Materials. Erbstatin was isolated from *Streptomyces* as described before (9). The A431 cell line was obtained from the late Dr. S. Kawai, Institute of Medical Science, University of Tokyo.

0006-291X/90 $1.50

EGF was purchased from Biomeidcal Technologies; and ATP, from Sigma. [^{3}H]Myo-inositol (18.3 Ci/mmol), [^{3}H]PtdIns 4,5-P_2 (1.0 Ci/mmol) and Amprep SAX column were obtained from Amersham.

Assay of inositol phosphate formation. A431 cells ($3x10^5$/well) grown for 16 hr beforehand in 24-well plates were prelabelled with [^{3}H]myo-inositol (1 μCi/ml) for 24 hr in inositol-free DMEM containing 10% dialyzed serum. Then, the medium was removed and cells were preincubated for 15 min in 0.5 ml of inositol-free DMEM containing 30 mM LiCl. Erbstatin was then added, and after 15 min EGF (400 ng/ml) was added, and incubation was continued and cells removed at specified intervals. The reaction was terminated by the additon of ice-cold 10% $HClO_4$, and the mixture was then neutralized by addition of 1.53 M KOH in 75 mM Hepes. The solution was kept on ice for 15 min, after which it was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was applied onto an Amprep SAX column, and the column was then washed with water and eluted with 0.17 M $KHCO_3$. The obtained eluate was counted in a liquid scintillation counter.

In vitro phospholipase C assay. The cells were collected, homogenized in buffer A (20 mM Hepes [pH 7.2], 30 mM sodium pyrophosphate, 50 mM sodium chloride, 5 mM β-glycerophosphate, 1 mM EGTA, 1 mM PMSF, 100 μM Na_3VO_4, 10 μg/ml of leupeptin, and 10 μg/ml of aprotinin), and fractionated into cytosolic and membrane fractions by ultracentrifugation at 100,000 xg for 30 min. The reaction buffer consisted of 15 μl of buffer A, 20 μl of 50 mM sodium phosphate (pH 6.8)-1 mM EGTA-100 mM potassium chloride, 5 μl of 8 mM calcium chloride, and 5 μl of [^{3}H]PtdIns 4,5-P_2. The reaction was started by addition of 5 μl of each fraction. The reaction mixture was incubated for 15 min at 37°C, and the reaction was stopped on ice by addition of 100 μl of 1% bovine serum albumin and 500 μl of 10% trichloroacetic acid. The precipitate was removed by centrifugation, and the release of [^{3}H]Ins 1,4,5-P_3 was measured with a liquid scintillation counter.

Results

EGF increased formation of total [^{3}H]-inositol phosphate 2- to 3-fold in 10 min, as shown in Fig. 1. Treatment of the cells with 50 μg/ml of erbstatin substantially repressed EGF-induced production of inositol phosphates, and this inhibition was dose-dependent (Fig. 2). The IC_{50} value was about 19 μg/ml. 5'-O-Methyl-erbstatin, an inactive analogue of erbstatin (10), did not inhibit EGF-induced inositol phosphate formation (Fig. 2). ATP-induced inositol phosphate production, which is considered to be not mediated by tyrosine phosphorylation (11), was not affected by erbstatin.

Next, we measured the *in vitro* phospholipase C activity of A431 cells that had been treated with EGF and/or erbstatin. Phospholipase C activity solubilized from EGF-treated A431 cells was 1.5 fold higher than that from untreated cells. However, in the presence of erbstatin, EGF failed to activate the enzyme (Fig. 3). In A431 cells, phospholipase C is located in both cytosolic and membrane compartments. In untreated cells, the cytosolic phospholipase C activity was higher than the membrane-bound one (Fig. 4). EGF activated only the cytosolic enzyme, and the

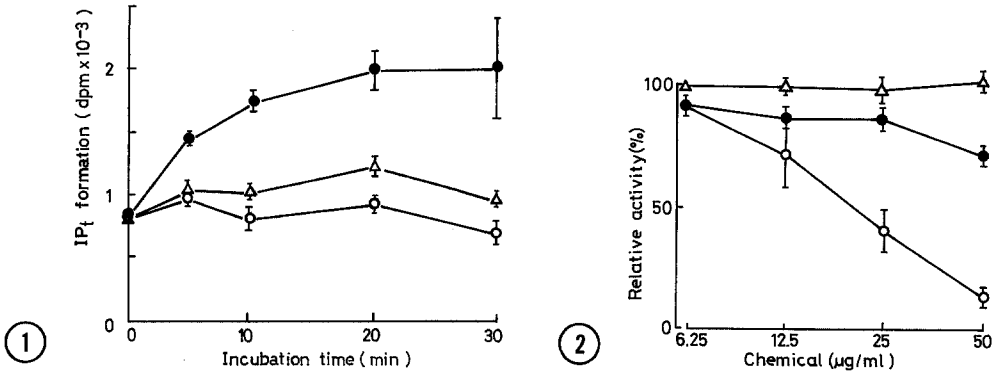

Fig. 1. Inhibition of EGF-induced inositol phosphate production by erbstatin in A431 cells. Inositol phosphates were extracted and separated on an anion-exchange column, and total inositol phosphates (IP + IP_2 + IP_3) were measured by liquid scintillation counting. Values are means±SD of triplicate samples. (○) No additive; (●) EGF; (△) EGF + erbstatin.

Fig. 2. Dose effect of erbstatin concentration on agonist-induced inositol phosphate production in A431 cells.
After pretreatment of A431 cells with erbstatin (○ , ●) or 5'-O-methyl-erbstatin (△) for 15 min at 37°C, EGF (400 ng/ml; ○ , △) or ATP (20 μM; ●) was added to the cells for 5 min. Total inositol phosphates were measured as described in "Materials and Methods". Values are means±SD of triplicate samples.

maximal increase was observed after 5 min. Subsequently, the amount of phospholipase C activity decreased, as shown in Fig. 4. This activation of cytosolic phospholipase C was inhibited by erbstatin.

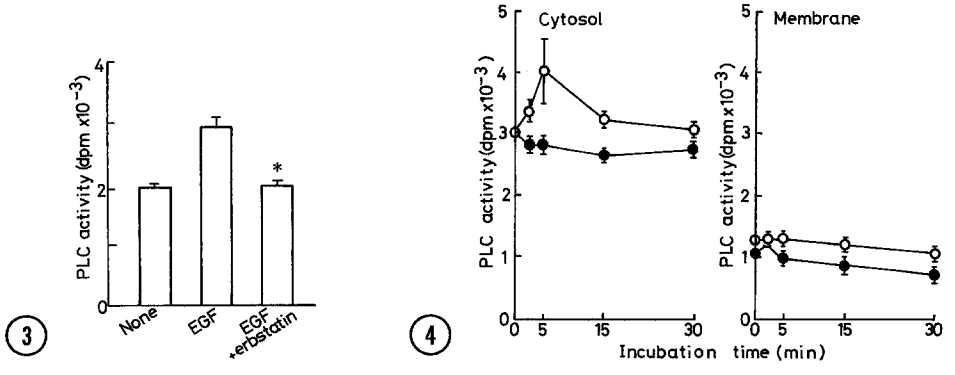

Fig. 3. Inhibition of EGF-induced whole phospholipase C activation by erbstatin.
A431 cells were treated or not with erbstatin (50 μg/ml) in the presence of Na_3VO_4 (100 μM) for 15 min, and then EGF (400 ng/ml) was added. After 5 min, the cells were collected, solubilized at 4°C for 30 min in buffer A containing 1% octylglucoside, and centrifuged at 14,500 xg for 30 min. The phospholipase C activity in the supernatant obtained was determined as described under "Materials and Methods". Values are means±SD of triplicate samples. * $P<0.02$.

Fig. 4. Effect of erbstatin pretreatment on cytosolic and membrane-bound phospholipase C's of A431 cells treated with EGF.
EGF (400 ng/ml) was added to A431 cells that had been pretreated (●) or not (○) with erbstatin (50 μg/ml) for 15 min; and after incubation for the indicated times, the cells were collected, homogenized, and fractionated into cytosolic and membrane fractions. The phospholipase C activity in both fractions was determined as described under "Materials and Methods". Values are means±SD of triplicate samples.

Discussion

Previously, we reported that erbstatin did not inhibit EGF-induced incorporation of inositol into phospholipids in A431 cells in Hepes-buffered saline (12). However, in this present study, we used inositol-free DMEM and observed the inhibition by erbstatin of inositol incorporation and of inositol phosphate formation. The reason for this discrepancy is due to a difference in the rate of penetration of the drug into cells in each medium (manuscript in preparation). In Hepes-buffered saline, erbstatin poorly penetrated into the cells. With use of the inositol-free DMEM, erbstatin inhibited the inositol phosphate formation at a concentration similar to that for inhibition of EGF receptor autophosphorylation in A431 cells (12). However, the drug did not inhibit ATP-induced inositol phosphate formation, in which G protein but not tyrosine kinase is involved in the activation (11). Therefore, it is likely that tyrosine kinase is involved in phospholipase C activation. We also found that erbstatin inhibits activation of phospholipase C by EGF.

Among the known phospholipase C isozymes, only phospholipase C-γ has been detected in A431 cells (11), and this enzyme is found in both cytosolic and membrane fractions (13). We found that only cytosolic phospholipase C was activated by EGF and that this activation was blocked by erbstatin. Therefore, it may be possible that only cytosolic phospholipase C is tyrosine-phosphorylated by EGF to be activated. This interpretation is supported by the observation that the phospholipase C activity immunoprecipitated from EGF-stimulated A431 cells by antiphosphotyrosine antibodies is cytosolic and not membrane bound (14).

Our results indicate that tyrosine kinase is funcitonally involved in EGF-induced phospholipase C activation and inositol phosphate formation.

Acknowledgments

This work was partly supported by grants from the Ministry of Health and Welfare, the Ministry of Education, Science, and Culture, the Foundation for Promotion of Cancer Research (Japan), and the Keio Technology Association. The authors wish to thank Miss M. Kubota for preparation of the manuscripts.

References

1. Hunter, T., and Cooper, J.A. (1985) Ann. Rev. Biochem. **54**, 897-930.
2. Berridge, M.I., and Irvine, R.F. (1984) Nature **312**, 315-321.
3. Nishizuka, Y. (1984) Nature **308**, 693-698.
4. Berridge, M.J., Heslop, J.P., Irvine, R.F., and Brown, K.D. (1984) Biochem. J. **222**,195-201.
5. Hepler, J.R., Nakahata, N., Lovenberg, T.W., DiGuiseppi, J., Herman, B., Earp, H.S., and Harden, T.K. (1987) J. Biol. Chem. **262**, 2951-2956.
6. Nishibe, S., Wahl, M.I., Rhee, S.G., and Carpenter, G.(1989) J. Biol. Chem. **264**,10335-10338.
7. Meisenhelder, J., Suh, P.-G., Rhee, S.G., and Hunter, T. (1989) Cell **57**, 1109-1122.
8. Wahl, M.I., Olashaw, N.E., Nishibe, S., Rhee, S.G., Pledger, W.J., and Carpenter, G. (1989) Mol. Cell. Biol. **9**, 2934-2943.
9. Umezawa, H., Imoto, M., Sawa, T., Isshiki, K., Matsuda, N., Uchida, T., Iinuma, H., Hamada, M., and Takeuchi, T. (1986) J. Antibiot. **39**, 170-173.
10. Isshiki, K., Imoto, M., Sawa, T., Umezawa, K., Takeuchi, T., and Umezawa, H. (1987) J. Antibiot. **40**, 1209-1210.
11. Wahl, M.I., Nishibe, S., Suh, P.-G., Rhee, S.G., and Carpenter, G. (1989) Proc. Natl. Acad. Sci. USA **86**, 1568-1572.
12. Imoto, M., Umezawa, K., Sawa, T., Takeuchi, T., and Umezawa, H. (1987) Biochem. International **15**, 989-995.
13. Rhee, S.G., Suh, P.-G., Ryu, S.-H., and Lee, S.Y. (1989) Science **244**, 546-550.
14. Wahl, M.I., Daniel, T.O., and Carpenter, G. (1988) Science **241**, 968-970.