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Tyrosine Kinase Regulates Phospholipase D Activation at a Point Downstream From Protein Kinase C in Osteoblast-Like Cells

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Abstract It has recently been shown that the activation of protein kinase C (PKC) induces protein tyrosine phosphorylation in osteoblast-like MC3T3-E1 cells. We previously reported that the activation of PKC stimulates phosphatidylcholine-hydrolyzing phospholipase D in these cells. In this study, we examined whether protein tyrosine kinase is involved in the PKC-induced activation of phospholipase D in MC3T3-E1 cells. Genistein, an inhibitor of protein tyrosine kinases, which by itself had little effect on choline formation, significantly suppressed the formation of choline induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of PKC, in a dose-dependent manner. Tyrphostin, an inhibitor of protein tyrosine kinases chemically distinct from genistein, also dose-dependently suppressed the TPA-induced formation of choline. Sodium orthovanadate, an inhibitor of protein tyrosine phosphatases, significantly enhanced the TPA-induced formation of choline in a dose-dependent manner. These results strongly suggest that protein tyrosine kinase regulates phospholipase D activity at a point downstream from PKC in osteoblast-like cells.

Key words: phospholipase D, protein kinase C, tyrosine kinase, osteoblast, TPA

It is well recognized that protein kinase C (PKC), which is activated by diacylglycerol resulting from the receptor-mediated hydrolysis of phosphatidylinositol, plays a pivotal role in cellular responses to various agonists including hormones, neurotransmitters and growth factors [Nishizuka, 1992]. In a previous study [Kozawa et al., 1989], we showed that PKC is involved in the process that directs osteoblast-like MC3T3-E1 cells toward proliferation and suppresses alkaline phosphatase activity, a marker of mature osteoblast phenotype [Peck et al., 1964; Stein et al., 1990] in these cells. Phosphatidylcholine is well-known to be hydrolyzed by phospholipase D, resulting in the formation of phosphatidic acid, which sequentially hydrolyzed to diacylglycerol. Nowadays, it is accepted that phospholipase D takes part in the activation of PKC and plays an important role in modulating cellular functions, since phosphatidylcholine is the principal phospholipid in cell membranes [Exton, 1990; Billah and Anthes, 1990; Zeisel, 1993]. The activation of phospholipase D has been reported to be both dependent on PKC and independent of PKC [Zeisel, 1993]. In a previous study [Kozawa et al., 1994], we have demonstrated that 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a PKC activating phorbol ester [Nishizuka, 1986], stimulates phospholipase D through the activation of PKC in osteoblast-like MC3T3-E1 cells and that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a potent osteoblastic mitogen [Hakeda et al., 1987], stimulates phospholipase D independent of PKC activation resulting from phosphoinositide hydrolysis in these cells.

Protein tyrosine kinase activity is known to be associated with oncogene products of the retroviral *src* gene family and with cell surface receptors for several growth factors [Hunter and Cooper, 1985; Hanks et al., 1988]. It is well recognized that protein tyrosine phosphorylation plays a crucial role for cell proliferation and transformation [Hunter and Cooper, 1985; Hanks et al., 1988]. In osteoblasts, it has recently been reported that the activation of PKC

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induces protein tyrosine phosphorylation in MC3T3-E1 cells [Quarles et al., 1993]. In the present study, we examined the involvement of tyrosine kinase in the activation of phosphatidylcholine-hydrolyzing phospholipase D induced by PKC in MC3T3-E1 cells. Herein, we show that protein tyrosine kinase regulates phospholipase D activity at a point downstream from PKC in osteoblast-like cells.

MATERIALS AND METHODS

Materials

[Methyl-^{3}H]Choline chloride (85 Ci/mmol) was purchased from Amersham Japan (Tokyo, Japan). TPA and sodium orthovanadate (vanadate) were purchased from Sigma Chemical Co. (St. Louis, MO). Genistein and tyrphostin were purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. TPA and genistein were dissolved in dimethylsulfoxide (DMSO). The maximum concentration of DMSO in the culture medium was 0.1%, and this did not affect the assay for measurement of the formation of choline.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983] were generously provided by Dr. M. Kumegawa (Meikai University, Sakado, Japan) and maintained in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4) were seeded into 35-mm diameter dishes in 2 ml of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

Measurement of the Formation of Choline

To determine phosphatidylcholine-hydrolyzing phospholipase D activity in osteoblast-like MC3T3-E1 cells, the cultured cells were labeled with [methyl-^{3}H]choline chloride (2 μCi/dish) for 24 h. The labeled cells were washed twice with 1 ml of an assay buffer [5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$] and subsequently incubated in 1 ml of the assay buffer containing 0.01% bovine serum albumin (BSA) at 37°C for 20 min. The cells were then stimulated by TPA. The reaction was terminated by adding 0.75 ml of ice-cold methanol. The dishes were stood on ice for 10 min. The contents were transferred to tubes to which chloroform was added, and stood on ice for 60 min. Chloroform and water were then added to a final ratio of 1:1:0.9 (chloroform–methanol–water). The tubes were centrifuged at 14,000*g* for 5 min, and the upper aqueous methanolic phase was taken for analysis of the water-soluble choline-containing metabolites. The methanolic phase was separated on a 1 ml of Dowex 50-WH$^+$ column as described [Cook and Wakelam, 1989] with a minor modification. In brief, the phase was diluted to 5 ml with water and applied to the column. Glycerophosphocholine and choline phosphate were removed with 24 ml of water, and choline was eluted with 8 ml of 1 M HCl. When indicated, the cells were pretreated with genistein, tyrphostin or vanadate for 20 min.

Determination

The radioactivity of ^{3}H samples was determined with a Beckman LS-6000IC liquid scintillation spectrometer.

Statistical Analysis

The data were analyzed by Student's *t*-test, and $P < 0.05$ was considered significant. All data are presented as the mean ± SD of triplicate determinations.

RESULTS

Effect of Genistein on TPA-Induced Formation of Choline in MC3T3-E1 Cells

In a previous study [Kozawa et al., 1994], we have shown that the stimulatory effect of TPA on the formation of choline is mediated through the activation of PKC in osteoblast-like MC3T3-E1 cells. In this study, we first examined the effect of genistein, an inhibitor of protein tyrosine kinases [Akiyama et al., 1987]. Genistein (30 μg/ml), which by itself had little effect on choline formation, significantly inhibited the TPA-induced formation of choline (Fig. 1). The effect of genistein was dose dependent in the range of 0.1–30 μg/ml (Fig. 2). The maximum effect of genistein on the TPA-induced formation of choline was observed at 30 μg/ml.

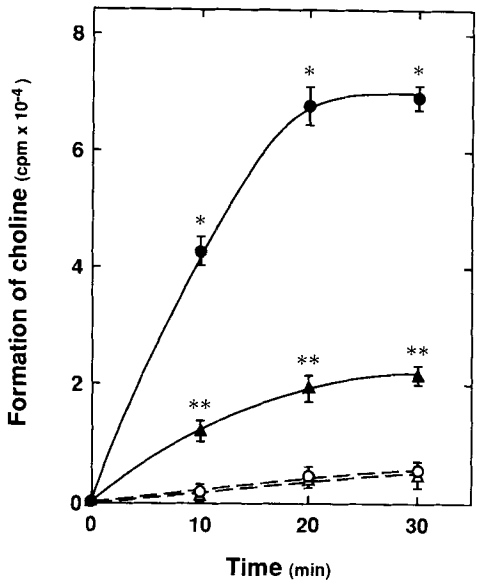

Fig. 1. Time-dependent effect of genistein on TPA-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with 30 μg/ml genistein (▲, △) or vehicle (●, ○) for 20 min and then stimulated by 0.1 μM TPA (▲, ●) or vehicle (△, ○) for the indicated periods. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the control value. **$P < 0.05$ compared to the value stimulated by TPA without genistein pretreatment.

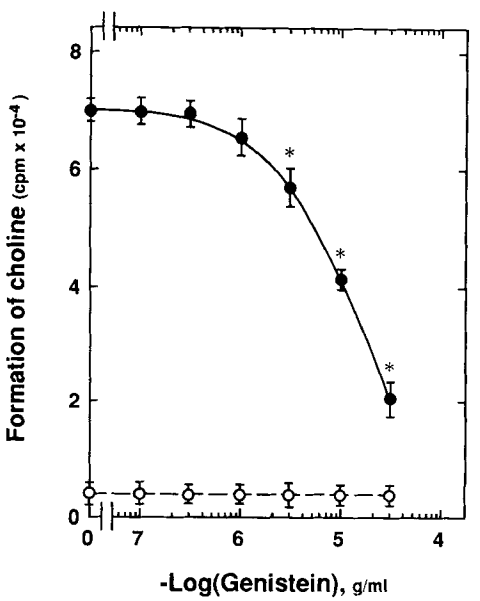

Fig. 2. Dose-dependent effect of genistein on TPA-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with various doses of genistein for 20 min and then stimulated by 0.1 μM TPA (●) or vehicle (○) for 20 min. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the control value.

Effect of Tyrphostin on TPA-Induced Formation of Choline in MC3T3-E1 Cells

To clarify the involvement of tyrosine kinase in the inhibitory effect of genistein on the TPA-induced phospholipase D activation in MC3T3-E1 cells, we next examined the effect of tyrphostin, another type of tyrosine kinase inhibitor [Gazit et al., 1989], on the TPA-induced formation of choline. The pretreatment with tyrphostin, which by itself had little effect on choline formation, also inhibited the TPA-induced formation of choline dose-dependently in the range of 10–100 μM (Fig. 3).

Effect of Vanadate on TPA-Induced Formation of Choline in MC3T3-E1 Cells

Furthermore, to clarify the role of tyrosine kinase in the TPA-induced phospholipase D activation in MC3T3-E1 cells, we examined the effect of vanadate, an inhibitor of protein tyrosine phosphatases [Klarlund et al., 1988]. Pretreatment with vanadate (0.1 mM), which by itself had little effect on choline formation, significantly enhanced the TPA-induced formation of choline (Figs. 4, 5). The effect of vanadate was dose dependent in the range of 1 μM–0.1 mM (Fig. 5). The maximum effect of vanadate on the TPA-induced formation of choline was observed at 0.1 mM.

DISCUSSION

We previously reported that TPA, a PKC activating phorbol ester [Nishizuka, 1986], significantly stimulates phospholipase D activity through the activation of PKC in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1994]. In the present study, we demonstrated that genistein, a tyrosine kinase inhibitor [Akiyama et al., 1987], inhibited the TPA-induced formation of choline in a dose-dependent manner in these cells. So, it seems that protein tyrosine kinase is involved in phospholipase D activation by PKC in MC3T3-E1 cells. In addition, we showed that tyrphostin, which is an inhibitor of tyrosine kinase chemically distinct from genistein [Gazit et al., 1989], also inhibited the TPA-induced choline formation. Thus, these results suggest that the suppression by genistein and tyrphostin of the PKC-induced phospholipase D activity is mediated through the inhibition of tyrosine kinase. Furthermore, vanadate, an inhibitor of protein tyrosine phosphatases [Klarlund et al.,

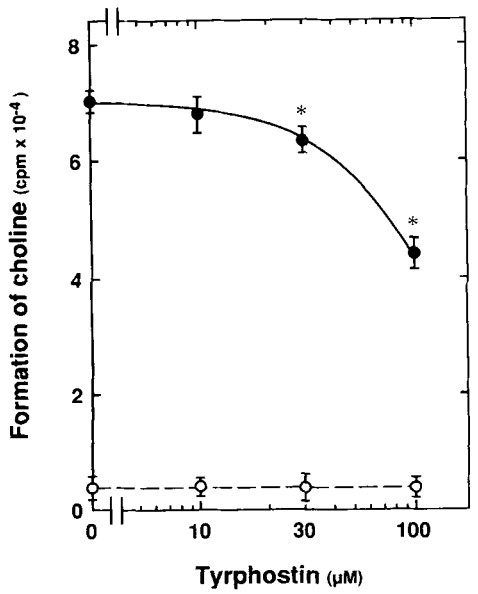

Fig. 3. Effect of tyrphostin on TPA-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with various doses of tyrphostin for 20 min and then stimulated by 0.1 μM TPA (●) or vehicle (○) for 20 min. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the control value.

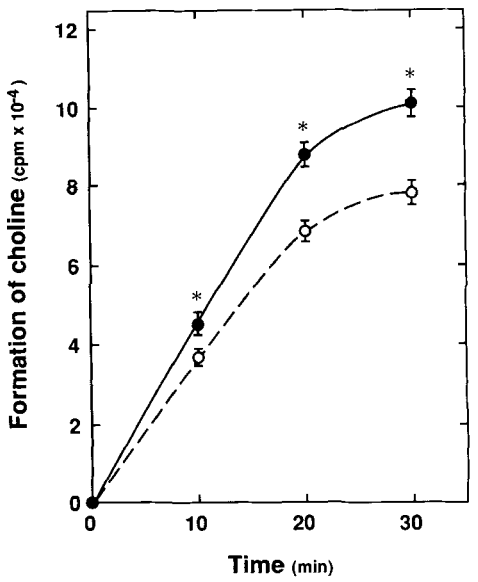

Fig. 4. Time-dependent effect of vanadate on TPA-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with 0.1 mM vanadate (●) or vehicle (○) for 20 min and then stimulated by 0.1 μM TPA for the indicated periods. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the value without vanadate pretreatment.

1988], markedly enhanced the TPA-induced formation of choline. It has recently been reported that the activation of PKC induces protein tyrosine phosphorylation in MC3T3-E1 cells [Quarles et al., 1993]. From these findings as a whole, it is most likely that PKC stimulates phospholipase D via protein tyrosine phosphorylation in osteoblast-like MC3T3-E1 cells. In other words, protein tyrosine kinase regulates phospholipase D activity at a point downstream from PKC.

It is well recognized that the activation of phospholipase D is dependent on PKC or independent of PKC [Nishizuka, 1992; Zeisel, 1993]. In a previous study [Kozawa et al., 1994], we have demonstrated that the TPA-induced activation of phospholipase D is inhibited by staurosporine, an inhibitor of protein kinases [Tamaoki et al., 1986] in MC3T3-E1 cells. Our finding suggests that TPA stimulates phospholipase D through the protein phosphorylation by PKC in these cells. However, it remains unclear whether phospholipase D is directly phosphorylated by PKC [Nishizuka, 1992]. It has recently been reported that tyrosine phosphorylation is involved in receptor coupling to phospholipase D, but the phospholipase D activity induced by phorbol 12,13-dibutyrate, a PKC-activating phorbol ester, is not inhibited by tyrosine kinase inhibitors in human neutrophils [Uings et al., 1992]. Since we here show that tyrosine kinase inhibitors suppressed the TPA-induced phospholipase D activity in osteoblast-like MC3T3-E1 cells, this discordance may be due to the difference of cell species. Further investigation would be required to clarify details.

Protein tyrosine kinases are well-known to be associated with the products of retroviral oncogenes exemplified with v-*src*, and the fundamental response of growth factor receptors [Hunter and Cooper 1985, Hanks et al., 1988]. It has recently been shown that v-*src* activates phospholipase D via independent pathway from PKC activation in BALB/c 3T3 fibroblasts [Song and Foster, 1993]. In the present study, we showed that PKC induces phospholipase D activation through protein tyrosine phosphorylation in osteoblast-like MC3T3-E1 cells. Accumulating evidence suggests that phospholipase D plays an important role in the intracellular signaling system which requires the activation of PKC [Exton, 1990; Billah and Anthes, 1990; Zeisel, 1993]. We previously showed that PKC is involved in the process which directs MC3T3-E1 cells toward proliferation [Kozawa et al., 1989], and it

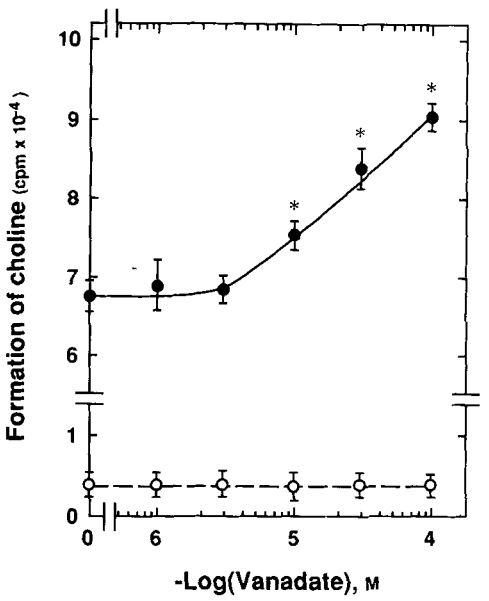

Fig. 5. Dose-dependent effect of vanadate on TPA-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with various doses of vanadate for 20 min and then stimulated by 0.1 μM TPA (●) or vehicle (○) for 20 min. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the control value.

has been demonstrated that protein tyrosine phosphorylation is involved in the proliferation of these cells [Quarles et al., 1993]. Therefore, these findings suggest that both tyrosine kinase activation and PKC activation act crucial roles in the proliferation of osteoblast-like MC3T3-E1 cells.

In conclusion, our results strongly suggest that protein tyrosine kinase regulates phospholipase D activity at a point downstream from PKC in osteoblast-like cells.

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REFERENCES

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S-I, Itoh N, Shibuya M, Fukami Y (1987): Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262:5592–5595.

Billah MM, Anthes JC (1990): The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem J 269:281–291.

Cook SJ, Wakelam MJO (1989): Analysis of the water-soluble products of phosphatidylcholine breakdown by ion-exchange chromatography. Biochem J 263:581–587.

Exton JH (1990): Signaling through phosphatidylcholine breakdown. J Biol Chem 265:1–4.

Gazit A, Yaish P, Gilon C, Levitzki A (1989): Tyrphostins I: Synthesis and biological activity of protein tyrosine kinase inhibitors. J Med Chem 32:2344–2352.

Hakeda Y, Hotta T, Kurihara N, Ikeda E, Maeda N, Yagyu Y, Kumegawa M (1987): Prostaglandin E_1 and $F_{2\alpha}$ stimulate differentiation and proliferation, respectively, of clonal osteoblastic MC3T3-E1 cells by different second messengers in vitro. Endocrinology 121:1966–1974.

Hanks SK, Quinn AM, Hunter T (1988): The protein kinase Family: Conserved features and deduced phylogeny of the catalytic domains. Science 241:42–52.

Hunter T, Cooper JA (1985): Protein-tyrosine kinases. Annu Rev Biochem 54:897–930.

Klarlund JK, Latini S, Forchhammer J (1988): Numerous proteins phosphorylated on tyrosine and enhanced tyrosine kinase activities in vanadate-treated *NIH 3T3* fibroblasts. Biochim Biophys Acta 971:112–120.

Kodama H, Amagai Y, Sudo H, Kasai S, Yamamoto S (1981): Establishment of a clonal osteoblastic cell line from newborn mouse calvaria. Jpn J Oral Biol 23:899–901.

Kozawa O, Suzuki A, Kotoyori J, Tokuda H, Watanabe Y, Ito Y, Oiso Y (1994): Prostaglandin $F_{2\alpha}$ activates phospholipase D independently from activation of protein kinase C. J Cell Biochem 55:375–379.

Kozawa O, Takatsuki K, Kotake K, Yoneda M, Oiso Y, Saito H (1989): Possible involvement of protein kinase C in proliferation and differentiation of osteoblast-like cells. FEBS Lett 243:183–185.

Nishizuka Y (1986): Studies and perspectives of protein kinase C. Science 233:305–312.

Nishizuka Y (1992): Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607–614.

Peck WA, Birge SJ Jr, Fedak SA (1964): Bone cells: Biochemical and biological studies after enzymatic isolation. Science 146:1476–1477.

Quarles LD, Haupt DM, Davidai G, Middleton JP (1993): Prostaglandin $F_{2\alpha}$-induced mitogenesis in MC3T3-E1 osteoblasts: Role of protein kinase-C-mediated tyrosine phosphorylation. Endocrinology 132:1505–1513.

Song J, Foster DA (1993): v-*src* activates a unique phospholipase D activity that can be distinguished from the phospholipase D activity activated by phorbol esters. Biochem J 294:711–714.

Stein GS, Lian JB, Owen TA (1990): Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 4:3111–3123.

Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S (1983): In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96:191–198.

Tamaoki T, Nomoto H, Kato Y, Morimoto M, Tomita F (1986): Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem Biophys Res Commun 135:397–402.

Uings IJ, Thompson NT, Randall RW, Spacy GD, Bonser RW, Hudson AT, Garland LG (1992): Tyrosine phosphorylation is involved in receptor coupling to phospholipase D but not phospholipase C in the human neutrophil. Biochem J 281:597–600.

Zeisel SH (1993): Choline phospholipids: Signal transduction and carcinogenesis. FASEB J 7:551–557.