Journal of Cellular Biochemistry 63:491–499 (1996)

Basic Fibroblast Growth Factor Stimulates Phosphatidylcholine-Hydrolyzing Phospholipase D in Osteoblast-Like Cells

Atsushi Suzuki, Junji Shinoda, Shigeru Kanda, Yutaka Oiso, and Osamu Kozawa

First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466, Japan (A.S., J.S., Y.O.); Ludwig Institute for Cancer Research, Biomedical Center, S-751 24 Uppsala, Sweden (S.K., O.K.); Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi 480-03, Japan (O.K.).

Abstract We examined the effect of basic fibroblast growth factor (bFGF) on the activation of phosphatidylcholine-hydrolyzing phospholipase D in osteoblast-like MC3T3-E1 cells. bFGF stimulated both the formations of choline (EC_{50} was 30 ng/ml) and inositol phosphates (EC_{50} was 10 ng/ml). Calphostin C, an inhibitor of protein kinase C (PKC), had little effect on the bFGF-induced formation of choline. bFGF stimulated the formation of choline also in PKC down regulated cells. Genistein and methyl 2,5-dihydroxycinnamate, inhibitors of protein tyrosine kinases, significantly suppressed the bFGF-induced formation of choline. Sodium orthovanadate, an inhibitor of protein tyrosine phosphatases, enhanced the bFGF-induced formation of choline. In vitro kinase assay for FGF receptors revealed that FGF receptor 1 and 2 were autophosphorylated after FGF stimulation. bFGF dose-dependently stimulated DNA synthesis of these cells. These results strongly suggest that bFGF activates phosphatidylcholine-hydrolyzing phospholipase D through the activation of tyrosine kinase, but independently of PKC activated by phosphoinositide hydrolysis in osteoblast-like cells.

Key words: tyrosine kinase, protein kinase C, proliferation, osteoblasts

Fibroblast growth factors (FGFs) comprise a family of heparin-binding growth factors that include nine members. FGF-1 to 9 [Basilico and Mascatelli, 1992]. It is well known that basic FGF (bFGF or FGF-2) has mitogenic effects and affects differentiation of many types of cells including mesodermal and neuroectodermal cells [Gospodarowicz et al., 1987]. In bone tissues, it has been reported that bFGF is found in bone matrix and that cultured osteoblast-like cells produce bFGF [Globus et al., 1989; Baylink et al., 1993; Hurley et al., 1994]. In addition, bFGF expression in macrophages, osteoblasts, or chondrocytes is detected during all stages of fracture repair: stage 1, immediate injury response; stage 2, intramembranous ossification; stage 3, chondrogenesis; stage 4, endochondral ossification [Bolander, 1992]. Thus, it is now recognized that bFGF plays an important role in bone metabolism and fracture healing. It has been reported that bFGF stimulates proliferation of osteoblasts [Hurley et al., 1994]. In addition, bFGF has been reported to decrease the insulin-like growth factor-I synthesis in rat calvaria cultures, reduce alkaline phosphatase activity, osteocalcin mRNA level, and type I collagen mRNA level, which are markers of differentiation of osteoblasts, in osteoblasts, including mouse osteoblast-like MC3T3-E1 cells [Rodan et al., 1989; Canalis et al., 1993; Hurley et al., 1993]. As for intracellular signaling system, it has been reported that there are four structurally related high affinity receptors (FGF receptor 1 to 4) that have an intrinsic protein tyrosine kinase activity, and elicit tyrosine autophosphorylation of the receptor [Coughlin et al., 1988; Johnson and Williams, 1993]. Tyrosine phosphorylation creates high affinity sites for binding of proteins that contain *src* homology 2-domain [Cadena and Gill, 1992]. In the FGF receptor 1, two intracellular autophosphorylated tyrosine residues have been identified, at Y653 and Y766, respectively [Hou et al., 1993].

Received April 29, 1996; accepted June 21, 1996.

Address reprint requests to Osamu Kozawa, M.D., present address: Department of Pharmacology, Gifu University School of Medicine, GIFU 500, Japan.

After the phosphorylation of Y766, phospholipase C $(PLC)_\gamma$, which has two *src* homology 2-domains, binds to this tyrosine residue and PLC_γ can be activated following tyrosine phosphorylation [Burgess et al., 1990]. The Y766F mutant FGF receptor 1 could not activate PLC_γ, but still mediates a mitogenic signal [Mohammadi et al., 1992; Peters et al., 1992] and allows neurite outgrowth when expressed in PC-12 pheochromocytoma cells [Spivak-kroizman et al., 1994]. It has been reported that FGF stimulates phosphoinositide hydrolysis by PLC [Taylor et al., 1988], resulting in the production of diacylglycerol and inositol phosphates [Burgess et al., 1990; Berridge, 1993]. Among these products, it is well accepted that diacylglycerol is a physiological activator of protein kinase C (PKC) [Nishizuka, 1992]. However, the details of the intracellular signaling system of FGF have not been fully clarified yet.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC), resulting in the formation of phosphatidic acid, which is a precursor of diacylglycerol [Billah and Anthes, 1990; Exton, 1990]. Although it has been shown that PC hydrolysis by PLD is secondary to the activation of PKC, recent evidence suggests that PC-PLD is also activated via an independent pathway from PKC activation [Billah and Anthes, 1990; Exton, 1990]. Nowadays, it is recognized that PC-PLD plays a crucial role in modulating cellular functions that require long-term activation of PKC, since PC is the principal phospholipid in cell membranes [Nishizuka, 1992]. It has recently been reported that bFGF stimulates PC-PLD in baby hamster kidney fibroblasts and human umbilical vascular endothelial cells [Motoike et al., 1993; Ahmed et al., 1994]. In the present study, we examined the effect of bFGF on PC-PLD activity in osteoblast-like MC3T3-E1 cells. Herein, we show that bFGF activates PC-PLD through the activation of tyrosine kinase, but independently of PKC activated by phosphoinositide hydrolysis in these cells.

METHODS

Materials

bFGF was purchased from Boehringer Mannheim K.K. (Tokyo, Japan), and acidic FGF (aFGF or FGF-1) was a kind gift from Dr. Ralf F. Pettersson (Ludwig Institute for Cancer Research, Stockholm, Sweden). *myo*-[^{3}H]Inositol (90 Ci/mmol), [*methyl*-^{3}H]choline chloride (85 Ci/mmol), [^{3}H]PDBu (phorbol-12,13-dibutylate) (14.4 Ci/mmol), [*methyl*-^{3}H]thymidine (22 Ci/mmol), and [γ-^{32}P]ATP (3 Ci/mmol) were purchased from Amersham International (Buckinghamshire, UK). Calphostin C was purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Methyl 2,5-dihydroxycinnamate (cinnamate) was purchased from Life Technologies, Inc. (Gaithersburg, MD). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), PDBu, and sodium orthovanadate (vanadate) were purchased from Sigma Chemical Co. (St. Louis, MO). Other materials and chemicals were obtained from commercial sources. Calphostin C, TPA, genistein, and cinnamate were dissolved in dimethyl sulphoxide. The maximum concentration of dimethyl sulfoxide was 0.1% and this did not affect the assay for the formation of choline.

Antibody

Anti-FGF receptor 1 IgG was raised and affinity-purified as described [Wennström et al., 1991]. Anti-FGF receptor 2 and anti-FGF receptor 3 IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FGF receptor 4 antiserum [Partanen et al., 1991] was a kind gift from Dr. Kali Alitalo (Molecular and Cancer Laboratory, University of Helsinki, Helsinki, Finland).

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. In experiments for the formation of inositol phosphates, the medium was exchanged for 2 ml of inositol-free α-MEM containing 0.3% FCS. When indicated, the cells were pretreated with TPA for 36 h.

Measurement of the Formation of Choline

To determine PC-hydrolyzing PLD activity, 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 (HEPES), 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 bFGF. The reaction was terminated by adding 0.75 ml ice-cold methanol. The dishes were placed on ice for 10 min. The contents were transferred to tubes to which chloroform was added and placed on ice for 60 min. Chloroform and water were then added at 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 separated for analysis of the water-soluble choline-containing metabolites. Separation was conducted on a 1 ml Dowex 50-WH^+ column (200–400 mesh) 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. The radiolabeled choline was eluted with 8 ml of 1 M HCl. When indicated, the cells were pretreated with calphostin C, genistein, cinnamate, or vanadate for 20 min.

Measurement of the Formation of Inositol Phosphates

Measurement of the formation of inositol phosphates was performed as previously described [Suzuki et al., 1994]. The cultured cells were labeled with *myo*-[^{3}H]inositol (3 μCi/dish) for 48 h. The labeled cells were preincubated with 10 mM LiCl for 10 min in 1 ml of the assay buffer containing 0.01% BSA. The cells were then stimulated by bFGF. The reaction was terminated by adding 1 ml 30% trichloroacetic acid. The acid supernatant was treated with diethyl ether to remove the acid and neutralized with 0.1 M NaOH. The supernatant was applied to a 1 ml Dowex AG1-X8 column (100–200 mesh, formate form). The radioactive inositol phosphates were eluted from the column with 8 ml of 0.1 M formic acid containing 1 M ammonium formate.

Assay for [^{3}H]PDBu Binding

The assay for [^{3}H]PDBu binding was performed as previously described with minor modifications [Kikuchi et al., 1986]. In brief, the cultured cells with or without 36 h pretreatment by 0.1 μM TPA were washed twice with the assay buffer, and then incubated in 1 ml of α-MEM with 50 nM of [^{3}H]PDBu. The incubation was carried out for 20 min at 37°C and the reaction was terminated by aspirating the medium, and then immediately washed three times with the assay buffer. Nonspecific binding was determined by incubating the cells with [^{3}H]PDBu in the presence of 20 μM unlabeled PDBu. Nonspecific counts represented less than 25% of the total counts.

Measurement of DNA Synthesis

Measurement of DNA synthesis was performed as previously described [Suzuki et al., 1993]. In brief, the cultured cells were incubated with various doses of bFGF in 1 ml of α-MEM containing 0.3% FCS at 37°C for 28 h. Six hours before harvest, the cells were pulsed with [*methyl*-^{3}H]thymidine (0.5 μCi/dish). The incubation was terminated by adding 10% trichloroacetic acid and the radioactivity in the acid-insoluble materials was determined.

In Vitro Kinase Assay

The cultured cells in 100-mm diameter dishes were stimulated by aFGF (100 ng/ml) or bFGF (100 ng/ml) at 37°C for 8 min. The cells were rinsed with ice-cold Tris-buffered saline containing 500 μM vanadate and 1 mM dithiothreitol (DTT) and lysed in Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM EDTA, 500 μM vanadate, 1 mM DTT, 100 U/ml aprotinin). After a 15 min centrifugation at 15,000*g* at 4°C, the supernatant was used for immunoprecipitation with the indicated antibodies. The immune-complex was collected using protein A-Sepharose CL-4B, the beads were washed and resuspended in kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.05% Triton X-100, 1 mM DTT) and in vitro phosphorylation was performed in the presence of [γ-^{32}P]ATP for 10 min on ice and reaction was stopped by addition of 2 × sample buffer [8% sodium dodecyl sulfate (SDS), 0.4 M Tris-HCl, pH 8.8, 1 M sucrose, 10 mM EDTA, 0.02% bromophenol blue, 4% 2-mercaptoethanol]. The samples were heated at 95°C for 4 min and analyzed through SDS-gel electrophoresis in 5–12% gradient polyacrylamide gel. After fixation, the gel was treated with 1 M KOH for 30 min at 55°C, fixed again, dried, and exposed on RX films (Fuji, Japan).

Determination

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

Statistical Analysis

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

RESULTS

Effects of bFGF on Formations of Choline and Inositol Phosphates in MC3T3-E1 Cells

bFGF significantly stimulated the formation of choline up to 15 min in osteoblast-like MC3T3-E1 cells (Fig. 1). The effect of bFGF was dose-dependent in the range between 1 and 100 ng/ml (Fig. 2). The maximum effect of bFGF on the formation of choline was observed at 100 ng/ml. In addition, we found that bFGF dose dependently induced the formation of inositol phosphates (Fig. 2). The effect of bFGF on the formation of inositol phosphates (EC_{50} was 10 ng/ml) was more potent than that on the choline formation (EC_{50} was 30 ng/ml).

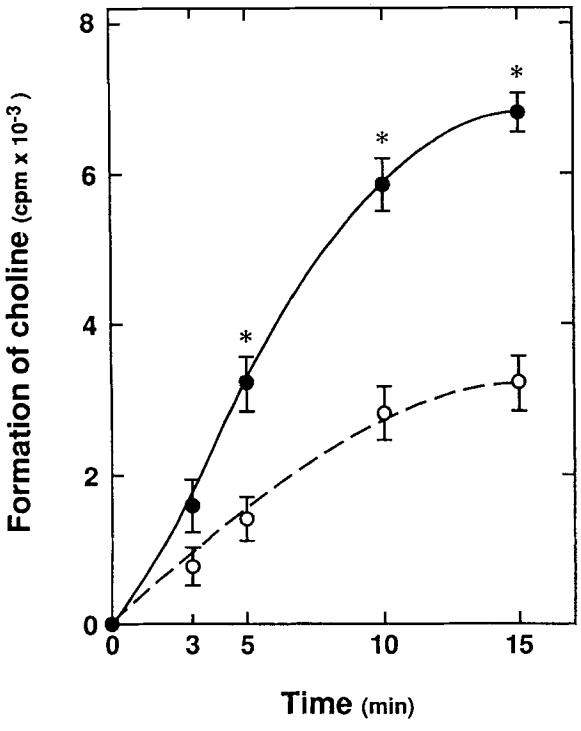

Fig. 1. Effect of bFGF on the formation of choline in MC3T3-E1 cells. The labeled cells were stimulated by 100 ng/ml bFGF (●) or vehicle (○) for the indicated periods. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. control values.

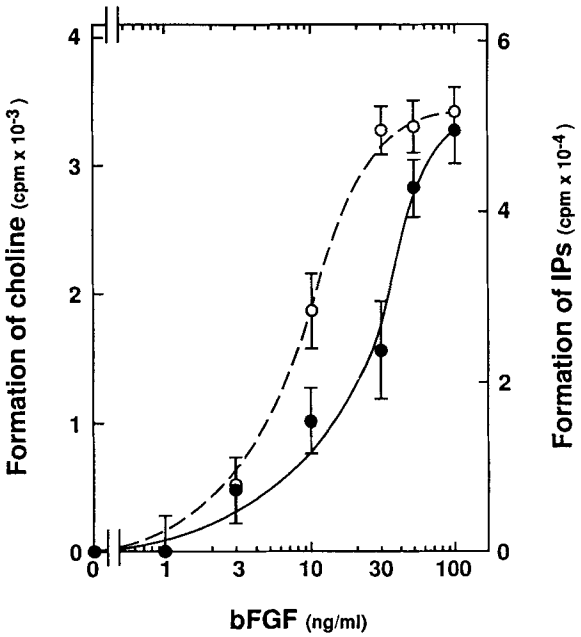

Fig. 2. Dose-dependent effects of bFGF on the formations of choline (●) and inositol phosphates (IPs) (○) in MC3T3-E1 cells. The labeled cells were stimulated by various doses of bFGF for 15 min. Values for control cells have been subtracted from each data point. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times.

In a previous study [Kozawa et al., 1994], we have shown that TPA stimulates PC-PLD through the activation of PKC in MC3T3-E1 cells. Calphostin C, a highly selective inhibitor of PKC [Kobayashi et al., 1989], which did not affect basal formation of choline, had little effect on the bFGF-induced formation of choline in MC3T3-E1 cells (Fig. 3). The long-term pretreatment with TPA had little effect on the formation of choline stimulated by bFGF in these cells (3,688 ± 221 cpm for control; 7,112 ± 356 cpm for 100 ng/ml bFGF alone; 3,499 ± 307 cpm for 0.1 μM TPA pretreatment; 6,997 ± 428 cpm for 100 ng/ml bFGF with 0.1 μM TPA pretreatment, as measured during the stimulation for 15 min). We confirmed that binding capacity of [^{3}H]PDBu in the cells with TPA long-term pretreatment was reduced to 27% of the capacity in intact cells (506.3 ± 16 fmol/10^6 cells for control; 143.9 ± 10 fmol/10^6 cells for the cells with 24 h pretreatment with 0.1 μM TPA, specific activity for 20 min).

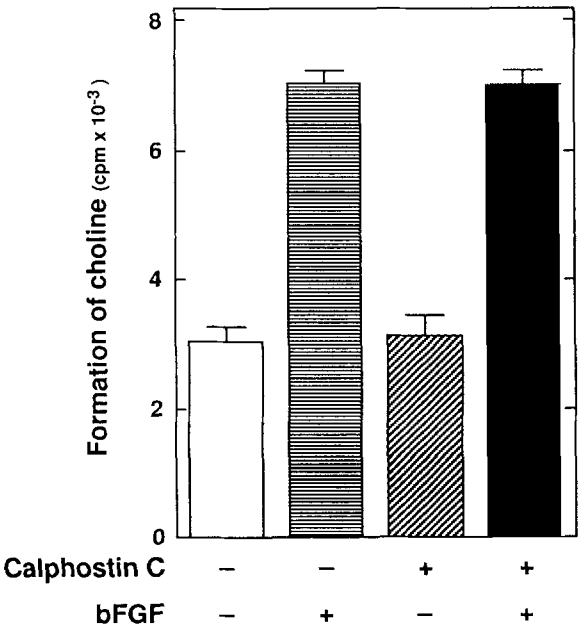

Fig. 3. Effect of calphostin C on the bFGF-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with 100 nM calphostin C or vehicle for 20 min, and subsequently stimulated by 100 ng/ml bFGF or vehicle for 15 min. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times.

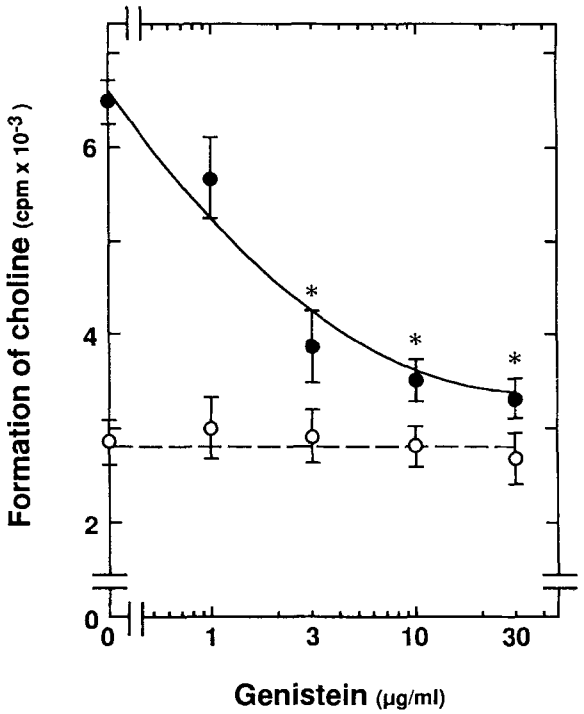

Fig. 4. Effect of genistein on the bFGF-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with various doses of genistein for 20 min, and subsequently stimulated by 100 ng/ml bFGF (●) or vehicle (○) for 15 min. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times. *P < 0.05 vs. value of bFGF alone.

Effects of Genistein and Cinnamate on Formation of Choline Induced by bFGF in MC3T3-E1 Cells

We examined the effect of genistein, an inhibitor of protein tyrosine kinases [Akiyama et al., 1987], on the bFGF-induced formation of choline in MC3T3-E1 cells. Genistein did not affect basal levels, but markedly inhibited the bFGF-induced formation of choline in MC3T3-E1 cells. This effect of genistein was dose-dependent in the range between 1 and 30 μg/ml (Fig. 4). The maximum effect of genistein was observed at 30 μg/ml. We next examined the effect of cinnamate, another tyrosine kinase inhibitor chemically distinct from genistein [Umezawa et al., 1990], on the bFGF-induced formation of choline. The pretreatment with cinnamate did not affect basal levels, but also suppressed the bFGF-induced formation of choline (Table I).

TABLE I. Effect of Cinnamate on bFGF-Induced Formation of Choline in MC3T3-E1 Cells⁺

	Choline formation (cpm)
Control	3,014 ± 211
bFGF	6,826 ± 411
Cinnamate + control	2,986 ± 301
Cinnamate + bFGF	3,876 ± 294*

⁺The labeled cells were pretreated with 30 μM cinnamate for 20 min, and then stimulated by 100 ng/ml bFGF for 15 min. Each value represents the mean ± S.E. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
*P < 0.05 compared to the value of bFGF alone.

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

To clarify the role of tyrosine kinase in the bFGF-induced formation of choline, we examined the effect of vanadate, an inhibitor of protein tyrosine phosphatases [Klarlund et al., 1988], on the choline formation induced by bFGF in MC3T3-E1 cells. Vanadate (30 μM) did not affect basal choline formation, but significantly enhanced the formation of choline induced by 30 ng/ml bFGF (Fig. 5).

In Vitro Kinase Assay for FGF Receptors in MC3T3-E1 Cells

There are four types of FGF receptors which have a tyrosine kinase domain and can trans-

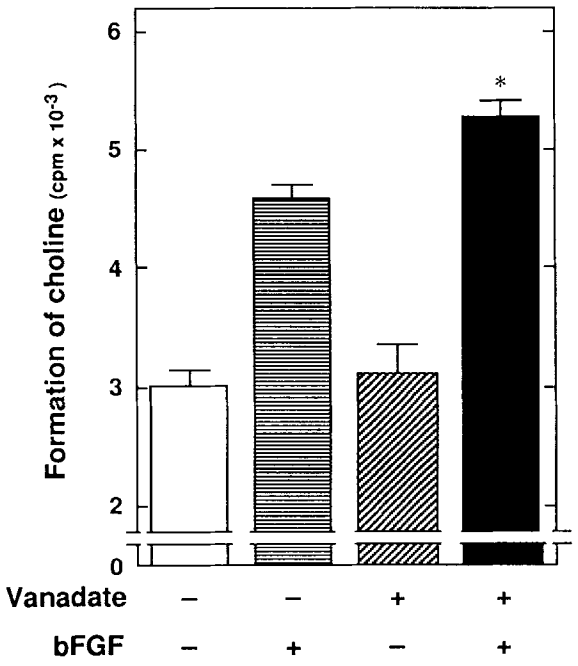

Fig. 5. Effect of vanadate on the bFGF-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with 30 μM vanadate for 20 min, and subsequently stimulated by 30 ng/ml bFGF or vehicle for 15 min. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. value of bFGF alone.

duce biological signals. To identify the receptor type that has activated PLD activity in MC3T3-E1 cells, in vitro kinase assay followed by immunoprecipitation using specific FGF receptor antibodies was performed. The reasons why this method was chosen are its high sensitivity and ability to identify the ligand-stimulated tyrosine kinase activity. FGF receptor 1 and 2 were autophosphorylated after both aFGF and bFGF stimulation, and phosphorylated FGF receptor 3 and 4 proteins were not observed after aFGF or bFGF stimulation (Fig. 6).

Effect of bFGF on DNA Synthesis of MC3T3-E1 Cells

We next examined the proliferative effect of bFGF on MC3T3-E1 cells. bFGF stimulated DNA synthesis of these cells in a dose-dependent manner in the range between 1 and 100 ng/ml (Fig. 7).

DISCUSSION

In the present study, we showed that the bFGF-induced formation of choline was inhibited by genistein in osteoblast-like MC3T3-E1 cells. Genistein is known to be a potent inhibitor of protein tyrosine kinases [Akiyama et al., 1987]. So it seems that protein tyrosine kinase is involved in regulating the bFGF-induced PC-PLD activation in MC3T3-E1 cells. In addition, we showed that cinnamate, which is an inhibitor of protein tyrosine kinases chemically distinct from genistein [Umezawa et al., 1990], also inhibited the bFGF-induced choline formation. Thus, these results suggest that the suppression by genistein and cinnamate of the bFGF-induced choline formation is mediated through the inhibition of tyrosine kinase. Furthermore, we demonstrated that vanadate, an inhibitor of protein tyrosine phosphatases [Klarlund et al., 1988], significantly enhanced the bFGF-induced formation of choline in MC3T3-E1 cells. Therefore, it is most likely that bFGF stimulates PC-PLD activity through the activation of tyrosine kinase in osteoblast-like MC3T3-E1 cells.

In a previous study [Kozawa et al., 1994], we have shown that TPA stimulates PC-PLD through the activation of PKC in MC3T3-E1 cells. In the present study, we demonstrated that bFGF stimulated both the formations of choline and inositol phosphates in these cells. It is well recognized that bFGF stimulates receptor-associated tyrosine kinase, and it has been shown that bFGF subsequently activates PLC_γ which hydrolyzes phosphoinositides [Taylor et al., 1988; Burgess et al., 1990]. Phosphoinositides are hydrolyzed by PLC resulting in the formation of diacylglycerol and inositol phosphates [Berridge, 1993]. Among these products, diacylglycerol is responsible for the activation of PKC. However, we found that calphostin C, a highly selective inhibitor of PKC [Kobayashi et al., 1989], had little effect on the bFGF-induced formation of choline. Furthermore, we demonstrated that long-term pretreatment with TPA had little effect on the bFGF-induced formation of choline in these cells. It has been reported that 24 h pretreatment with TPA down regulates PKC in MC3T3-E1 cells [Sakai et al., 1992], and we here confirmed that the binding capacity of PDBu in the cells with TPA long-term pretreatment was significantly reduced. So, it is unlikely that PKC activated by phosphoinositide hydrolysis is involved in the bFGF-induced formation of choline. Therefore, our findings suggest that bFGF stimulates PC-PLD independently from the activation of PKC in osteoblast-like MC3T3-E1 cells. It has recently been reported that PKC is responsible for bFGF-induced

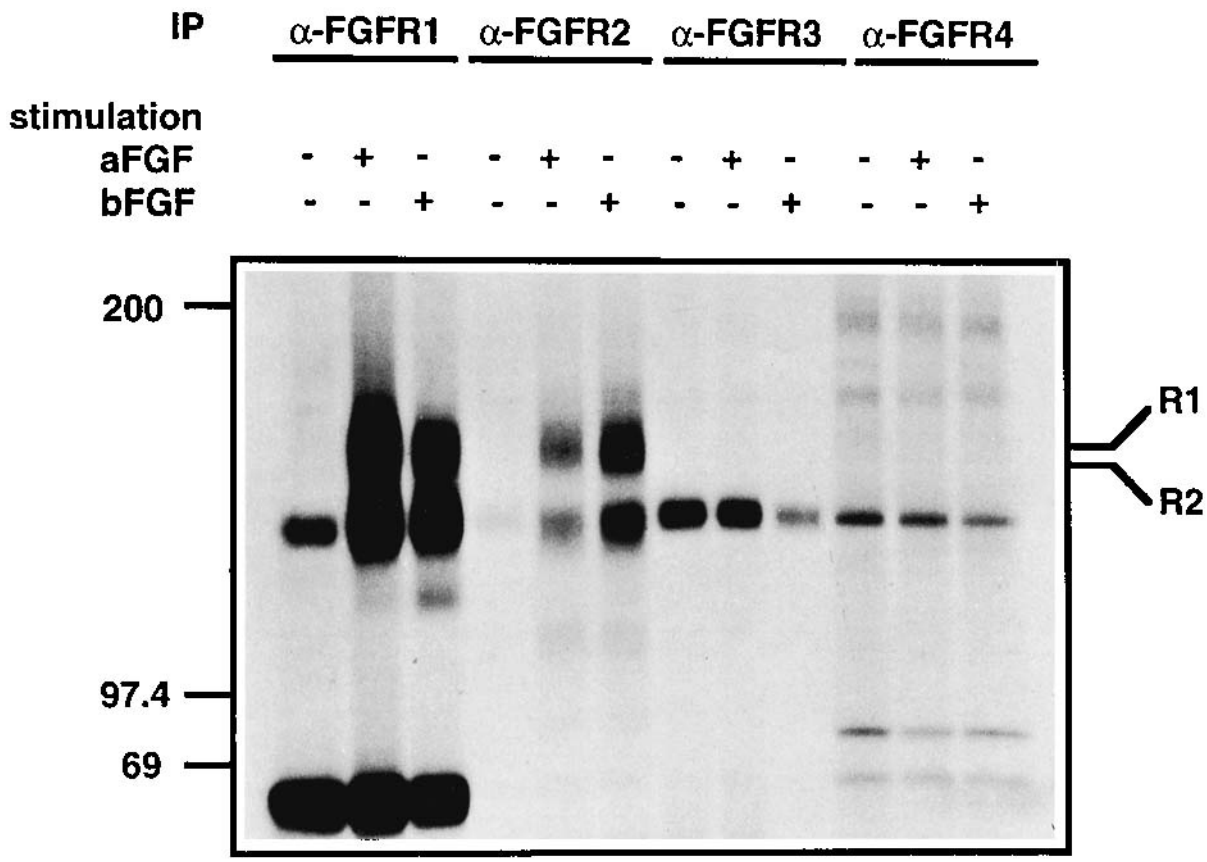

Fig. 6. Analysis of the expression of FGF receptors on MC3T3-E1 cells. The expression of FGF receptors was analyzed using in vitro kinase assay following immunoprecipitation (IP) with specific anti-FGF receptor (FGFR) antibodies, with or without stimulation of cells with aFGF (100 ng/ml) or bFGF (100 ng/ml). After precipitation of receptor proteins, [γ-^{32}P]ATP was incorporated into proteins and the samples separated by SDS-polyacrylamide gel electrophoresis, and then fixed, dried, and exposed. The numbers described on the left of the figure indicate the molecular weights (kDa). On the right, the migration position of the mature forms of FGF receptor 1 (R1) and FGF receptor 2 (R2) are indicated.

PLD activation in human umbilical endothelial cells [Ahmed et al., 1994]. The discordance may be due to the difference of cell type.

In this study, we identified the expression of functional FGF receptors 1 and 2. To identify the type of expressed FGF receptors, we used both aFGF and bFGF because of the difference of ligand affinity. By the alternative splicing, FGF receptors 1, 2, and 3 have several isoforms especially observed in the third immunoglobulin-like domain of the extracellular part of the receptors and the difference in this domain mainly affects the ligand affinity [Johnson and Williams, 1993]. In the case of FGF receptor 4, the isoform of this domain has not been reported yet [Partanen et al., 1992]. But using both aFGF and bFGF as a ligand for the detection of kinase activity, we could identify the expression of all 4 types of FGF receptors, because all isoforms reported are able to bind either aFGF or bFGF, or both FGFs. According to our results, MC3T3-E1 cells expressed kinase active FGF receptors 1 and 2. So, these findings suggest that PC-PLD activation by bFGF is transduced via FGF receptors 1 and/or 2 in MC3T3-E1 cells. Unfortunately, we could not identify which receptor could transduce the signal of PC-PLD activation. The transfection of the chimeric receptor into MC3T3-E1 cells, of which the extracellular part is changed to the other type of receptor that is not expressed on MC3T3-E1 cells, and the intracellular part is from FGF receptors 1 or 2, will give us an answer to this question.

It has been reported that bFGF stimulates the proliferation of osteoblasts [Baylink et al., 1993], and we showed here that bFGF stimulated the DNA synthesis of osteoblast-like MC3T3-E1 cells dose dependently. In a previous study [Kozawa et al., 1989], we have reported that PKC is

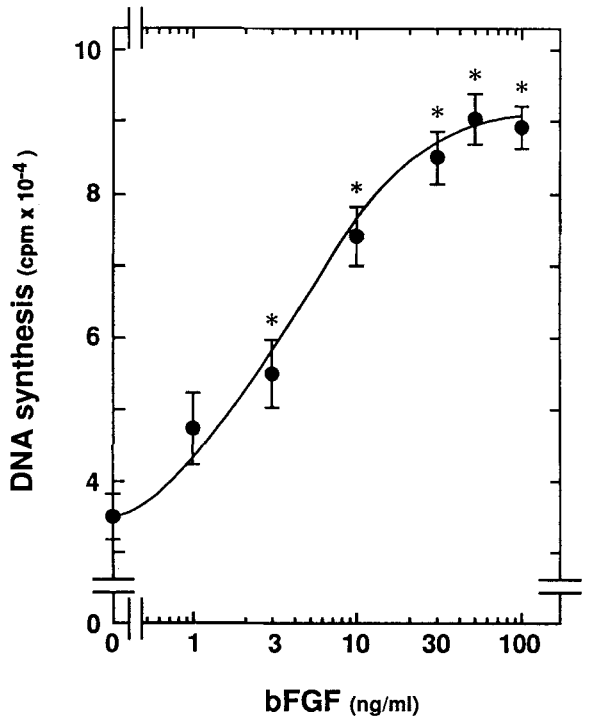

Fig. 7. Effect of bFGF on DNA synthesis of MC3T3-E1 cells. The cells were stimulated with various doses of bFGF for 28 h. Six hours before harvest, the cells were pulsed with [*methyl*-^{3}H]thymidine (0.5 μCi/dish). Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times.

involved in the process that directs osteoblast-like MC3T3-E1 cells toward proliferation. It is generally accepted that PKC is activated physiologically by diacylglycerol [Nishizuka, 1992]. Diacylglycerol is recognized to be produced not only from phosphoinositide hydrolysis by PLC, but also from PC hydrolysis by PLD [Billah and Anthes, 1990; Exton, 1990; Nishizuka, 1992]. In the present study, we demonstrated that bFGF stimulated both phosphoinositide hydrolysis by PLC and PC hydrolysis by PLD in MC3T3-E1 cells. Therefore, these results suggest that PKC activation through the stimulation of both PLC and PLD takes part in the bFGF-induced proliferation of osteoblast-like MC3T3-E1 cells.

In conclusion, our results strongly suggest that bFGF activates PLD through the activation of tyrosine kinase, but independently from the activation of PKC in osteoblast-like cells.

ACKNOWLEDGMENTS

We thank to Prof. Carl-Henrik Heldin for his enthusiastic encouragement. We are grateful to Dr. Lena Claesson-Welsh, Dr. Kali Alitalo, and Dr. Ralf F. Pettersson for giving us anti-FGF receptor 1 IgG, anti-FGF receptor 4 antiserum, and aFGF, respectively.

REFERENCES

Ahmed A, Plevin R, Shoaibi MA, Fountain SA, Ferriani RA, Smith SK (1994): Basic FGF activates phospholipase D in endothelial cells in the absence of inositol-lipid hydrolysis. Am J Physiol 266:C206–C212.

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

Basilico C, Mascatelli D (1992): The FGF family of growth factors and oncogenes. Adv Cancer Res 59:115–165.

Baylink DJ, Finkelman RD, Mohan S (1993): Growth factors to stimulate bone formation. J Bone Miner Res 8:S565–S572.

Berridge MJ (1993): Inositol trisphosphate and calcium signalling. Nature 361:315–325.

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

Bolander ME (1992): Regulation of fracture repair by growth factors. Proc Soc Exp Biol Med 200:165–170.

Burgess WH, Dionne CA, Kaplow J, Mudd R, Friesel R, Zilberstein A, Schlessinger J, Jaye M (1990): Characterization and cDNA cloning of phospholipase C-γ, a major substrate for heparin-binding growth factor 1 (acidic fibroblast growth factor)-activated tyrosine kinase. Mol Cell Biol 10:4770–4777.

Cadena DL, Gill GN (1992): Receptor tyrosine kinases. FASEB J 6:2332–2337.

Canalis E, Pash J, Gabbitas B, Rydziel S, Varghese S (1993): Growth factors regulate the synthesis of insulin-like growth factor-I in bone cell cultures. Endocrinology 133: 33–38.

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

Coughlin SR, Barr PJ, Cousens LS, Fretto LJ, Williams LT (1988): Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. J Biol Chem 263:988–993.

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

Globus RK, Plouet J, Gospodarowicz D (1989): Cultured bovine bone cells synthesize basic fibroblast growth factor and store it in their extracellular matrix. Endocrinology 124:1539–1547.

Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G (1987): Structural characterization and biological functions of fibroblast growth factor. Endocr Rev 8:95–114.

Hou J, Mckeehan K, Kan M, Carr SA, Huddleston MJ, Crabb JW, Mckeehan WL (1993): Identification of tyrosine 154 and 307 in the extracellular domain and 653 and 766 in the intracellular domain as phosphorylation sites in the heparin-binding fibroblast growth factor receptor tyrosine kinase (flg). Protein Sci 2:86–92.

Hurley MM, Abreu C, Harrisson JR, Lichtler AC, Raisz LG, Kream BE (1993): Basic fibroblast growth factor inhibits type I collagen gene expression in osteoblastic MC3T3-E1 cells. J Biol Chem 268:5588–5593.

Hurley MM, Abreu C, Gronowicz G, Kawaguchi H, Lorenzo J (1994): Expression and regulation of basic fibroblast growth factor mRNA levels in mouse osteoblastic MC3T3-E1 cells. J Biol Chem 269:9392–9396.

Johnson DE, Williams LT (1993): Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res 60:1–41.

Kikuchi A, Kozawa O, Hamamori Y, Kaibuchi K, Takai Y (1986): Inhibition of chemotactic peptide-induced phosphoinositide hydrolysis by phorbol esters through the activation of protein kinase C in differentiated human leukemia (HL-60) cells. Cancer Res 46:3401–3406.

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.

Kobayashi E, Nakano H, Morimoto M, Tamaoki T (1989): Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 159:548–553.

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, 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.

Kozawa O, Suzuki A, Kotoyori J, Tokuda H, Watanabe Y, Ito Y, Oiso Y (1994): Prostaglandin F_{α} activates phospholipase D independently from activation of protein kinase C in osteoblast-like cells. J Cell Biochem 55:375–379.

Mohammadi M, Dionne CA, Li W, Li N, Spivak T, Honegger AM, Jaye M, Schlessinger J (1992): Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. Nature 358:681–684.

Motoike T, Bieger S, Wiegandt H, Unsicker K (1993): Induction of phosphatidic acid by fibroblast growth factor in cultured baby hamster kidney fibroblasts. FEBS Lett 332: 164–168.

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

Partanen J, Makela TP, Eerola E, Korhonen J, Hirvonen H, Claesson-Welsh L, Alitalo K (1991): FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. EMBO J 10:1347–1354.

Partanen J, Vainikka S, Korhonen J, Armstrong E, Alitalo K (1992): Diverse receptor for fibroblast growth factors. Prog Growth Factor Res 4:69–83.

Peters KG, Marie J, Wilson E, Ives HE, Escobedo J, Rosario MD, Mirda D, Williams LT (1992): Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca^{2+} flux but not mitogenesis. Nature 358:678–681.

Rodan SB, Wesolowski G, Yoon K, Rodan GA (1989): Opposing effects of fibroblast growth factor and pertussis toxin on alkaline phosphatase, osteopontin, osteocalcin, and type I collagen mRNA levels in ROS 17/2.8 cells. J Biol Chem 264:19934–19941.

Sakai T, Okano Y, Nozawa Y, Oka N (1992): Different protein kinase C isoenzymes could modulate bradykinin-induced extracellular calcium-dependent and -independent increases in osteoblast-like MC3T3-E1 cells. Cell Calcium 13:329–340.

Spivak-kroizman T, Mohammadi M, Hu P, Jaye M, Schlessinger J, Lex I (1994): Point mutation in the fibroblast growth factor receptor eliminates phosphatidylinositol hydrolysis without affecting neuronal differentiation of PC12 cells. J Biol Chem 269:14419–14423.

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.

Suzuki A, Kotoyori J, Oiso Y, Kozawa O (1993): Prostaglandin E_2 is a potential mediator of extracellular ATP action in osteoblast-like cells. Cell Ad Commun 1:113–118.

Suzuki A, Kozawa O, Saito H, Oiso Y (1994): Effect of prostaglandin $F_{2\alpha}$ on Ca^{2+} influx in osteoblast-like cells. J Cell Biochem 54:487–493.

Taylor CW, Blakeley DM, Corps AN, Berridge MJ, Brown KD (1988): Effects of pertussis toxin on growth factor-stimulated inositol phosphate formation and DNA synthesis in Swiss 3T3 cells. Biochem J 249:917–920.

Umezawa K, Hori T, Tajima H, Imoto M, Isshiki K, Takeuchi T (1990): Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. FEBS Lett 260:198–200.

Wennström S, Sandstrom C, Claesson-Welsh L (1991): cDNA cloning and expression of a human FGF receptor which binds acidic and basic FGF. Growth Factors 4:197–208.