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Induction of phosphatidic acid by fibroblast growth factor in cultured baby hamster kidney fibroblasts

Toshiyuki Motoike[a], Sophie Bieger[a], Herbert Wiegandt[b], Klaus Unsicker[a,*]

[a]*Department of Anatomy and Cell Biology, University of Heidelberg, Im Neuenheimer Feld 307, D-69120 Heidelberg, Germany*
[b]*Department of Physiological Chemistry, University of Marburg, Karl-von-Frisch Straße, D-35043 Marburg, Germany*

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Basic fibroblast growth factor (bFGF/FGF-2) is a strong mitogenic inducer of cultured baby hamster kidney (BHK) cells. When cultured BHK cells were stimulated with FGF-2, phosphatidic acid (PA) was induced within 2 min, peaked at 5 min and gradually decreased. Phospholipase D (PLD) was also mitogenic for cultured BHK cells and this effect was mediated via PA. The possibility that PA induction by FGF-2 is an essential signaling step for BHK cell proliferation is discussed.

Fibroblast growth factor; Phosphatidic acid; Phospholipase D; BHK cell

1. INTRODUCTION

Many growth factors produce their signals by stimulating the hydrolysis of cellular phospholipids. A stimulation of the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2) to two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DG), has been demonstrated to occur in response to growth factors (for review, see [1,2]). Recently, it has been found that certain growth factors may stimulate the hydrolysis of phosphatidylcholine (PC), either alone or in conjunction with PIP2 hydrolysis. Evidence has accumulated showing that phosphatidic acid (PA) which is formed by phospholipase D (PLD)-catalysed hydrolysis of PC is implicated in the regulation of numerous cellular functions (for review, see [3,4]).

Basic fibroblast growth factor (FGF-2) is a member of a family consisting of seven closely related polypeptides with profound biological activities. FGF-2 is characterized as a potent modulator of cell proliferation, motility, differentiation and survival [5]. The receptors for FGFs were cloned and identified as a subgroup of the family of receptor tyrosine kinases [6]. The stimulation of such receptor tyrosine kinases leads to the association of enzymes, such as phospholipase C-γ1 (PLC-γ1) and phosphatidylinositol 3-kinase (PI3K) [7,8]. Although the activation of these particular enzymes appears to be essential for the initiation of a variety of cellular responses, mechanisms whereby these two enzymes transduce FGF-2-induced signals have not yet been fully clarified. Moreover, previous studies concerning the effects of FGF-2 on the modulation of second messengers such as phosphoinositide hydrolysis, intracellular Ca^{2+} mobilization and/or protein kinase C (PKC) activation have generated conflicting results [9–13]. The intracellular signals responsible for the cellular responses to FGF-2 in any target cells are incompletely understood.

In the case of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), it is now recognized that phosphatidylinositol turnover is not the only source of DG. PC breakdown has been reported to occur upon stimulation by these factors [14–17]. It is therefore very likely that FGF-2 also stimulates PC hydrolysis in some cells. This study was designed to elucidate whether FGF-2 induces PA accumulation in baby hamster kidney (BHK) cells. The possibility that PA is involved in the FGF-2-induced mitogenic signals is discussed.

*Corresponding author. Fax: (49) (6221) 56-5604.

Abbreviations: BHK cells, baby hamster kidney cells; DG, diacylglycerol; EGF, epidermal growth factor; FGF-2, β-fibroblast growth factor; IP3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PIPK, phosphatidylinositol-4-phosphate kinase; PI-PLC, phosphatidylinositol-specific phospholipase C; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC-γ1, phospholipase C-γ1; PLD, phospholipase D; RasGAP, Ras GTPase activating protein.

2. MATERIALS AND METHODS

2.1. *Materials*

Plastic culture dishes and 96-well plates were purchased from Greiner. Media components were from Gibco. PLD (*Streptomyces chromofuscus*, type VI) and dipalmitoyl PA were from Sigma. FGF-2

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was from Boehringer Mannheim. Silica gel plates (60, F-254) were from Merck. Other materials and chemicals were from commercial sources.

2.2. *Cell culture*

BHK cells were purchased from American Type Culture Collection. Cells were maintained at 37°C in 1:1 mixture of Dulbecco's modified Eagle's and Ham's F-12 media (DF-medium) supplemented with 5% fetal calf serum.

2.3. *Colorimetric proliferation assays*

BHK cells were seeded at 5×10^3 cells/wells of a 96-well microtiter plate. Twenty-four hours after seeding, cells were serum-starved with DF-medium containing 5 μg/ml transferrin and 100 μg/ml bovine serum albumin (BSA). FGF-2 or PLD were added to this serum-free medium at indicated concentrations. After 24 h, cell numbers were quantified employing the incorporation of Naphthol blue black into and subsequent elution from cells. Linearity of correlation between cell number and optical density was established previously [18]. Briefly, after 24 h treatment with FGF-2 or PLD, cells were fixed with formalin solution (10% formalin, 9% acetic acid and 0.1 M sodium acetate) for 15 min and stained with staining solution (0.1% Naphthol blue black, 9% acetic acid and 0.1 M sodium acetate) for 30 min. After washing with water, the dye was eluted with 50 mM NaOH. Absorbance of each well was measured at 620 nm with a reference wavelength of 492 nm with an ELISA reader (SLT Lab Instruments, Austria).

2.4. *Lipid extraction*

BHK cells were seeded at 6×10^5 cells/9 cm dish. Two days after seeding, subconfluent cells were serum-starved by washing 2 times with DF-medium containing 5 μg/ml transferrin and 100 μg/ml BSA and then incubated for 24 h at 37°C in the same medium. Quiescent cells were treated with FGF-2 or PLD at 37°C for the indicated time. The incubations were terminated by aspirating the medium, washing with phosphate-buffered saline and immediately adding 1.0 ml of ice-cold methanol. The cells were scraped and transferred to tubes and the dishes were further washed with 1.0 ml of ice-cold methanol. Then 1.0 ml of chloroform was added to the tubes and chloroform/methanol (1:2) suspensions were centrifuged (4,000 rpm × 10 min). The supernatant was saved and the pellet was reextracted with 2 ml of chloroform/methanol (1:1). To the combined supernatants, 1.0 ml of chloroform and 1.4 ml of water were added, mixed well and centrifuged (2,000 rpm × 5 min).

After removal of the upper layer, the lower layer was dried under N_2 and the residues were dissolved in 20 μl of chloroform and spotted on thin layer chromatography plates.

2.5. *Thin-layer chromatography of lipids*

PA was separated from other lipids essentially in the same way as described by Bocckino et al. [19]. The plates were developed with chloroform/methanol/water/acetic acid (65:25:4:1) to the top of the plate. The plates were dried and the top 3 cm of the plate containing the neutral lipids and phosphatidylethanolamine was removed. The plates were turned 180° and then developed with chloroform/methanol/ammonia (10:3:0.6) to the top of the plate two times. The plates were dried, stained with Coomassie brilliant blue R250 (0.03% in 30% methanol 100 mM NaCl) [20] for 30 min and scanned with a densitometer (Joyce Loebl-Chromoscan 3).

DG was separated using the solvent toluene/diethylether/ethanol/ammonia (50:40:2:0.2) and quantified as described above.

3. RESULTS

3.1. *Mitogenic effects of FGF-2 and PLD on BHK cells*

When BHK cells were treated with FGF-2, dose-dependent cell growth was observed. Exogenously added PLD also had a mitogenic effect on BHK cells, but this activity was not as potent as that of FGF-2 (Fig. 1).

3.2. *Time course of PA formation in response to FGF-2 and PLD*

The multidevelopment chromatography provided a clear separation of PA from other lipids in one dimension, and PA was well detected using Coomassie brilliant blue staining (Fig. 2). In FGF-2-treated BHK cells, PA was induced within 2 min, peaked at 5 min and then gradually decreased. In PLD-treated cells, PA was also induced, but more slowly. At the time point of 60 min, both FGF-2- and PLD- induced PA levels were still significantly higher than the control (Fig. 3).

3.3. *Time course of DG formation in response to FGF-2 and PLD*

In FGF-2-treated BHK cells, the DG content reached a maximum after 5 min and then decreased gradually. At the time point of 60 min, the DG level was already nearly the same as that of control. In contrast, in PLD-treated cells, only a slight increase in DG accumulation was observed (Fig. 4).

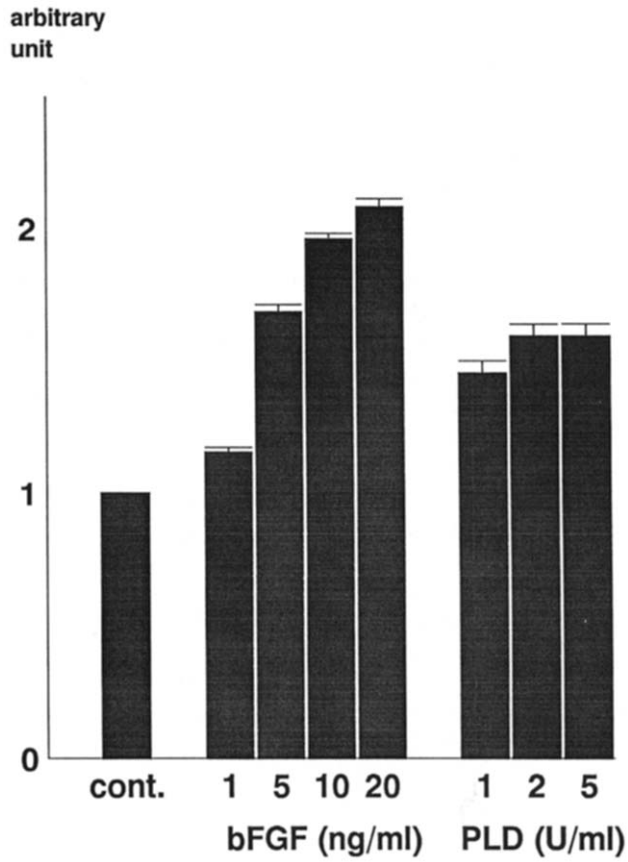

Fig. 1. Mitogenic effects of FGF-2 and PLD. The quantitated value of the untreated cells is expressed as 1.0 arbitrary unit. Results are mean standard deviation from 10 wells of two independent experiments.

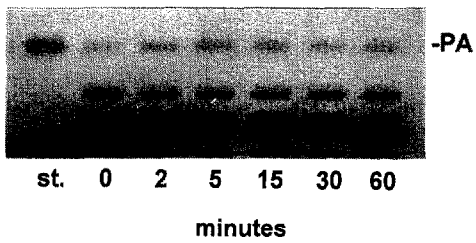

Fig. 2. Thin-layer chromatography of cell extracts and PA standard. Quiescent BHK cells were treated with FGF-2 (20 ng/ml) for the indicated time. Samples were processed and chromatographed as described in the text. st. = PA standard (dipalmitoyl PA).

4. DISCUSSION

BHK cells have high and low affinity binding sites for FGF-2. In addition, BHK cells themselves are capable of synthesizing FGF-2 ([21]; and Bieger S., unpublished observation). Therefore, it appears possible that endogenous FGF-2 may exert a mitogenic effect on BHK cells in autocrine or paracrine manners. Its mode of action, however, has not yet been fully clarified. In this study, we present PA as a possible second messenger in the FGF-2-induced mitogenic signaling pathways in BHK cells.

In the present study, the source of PA has not yet been identified. Formation of PA by FGF-2 could occur via several distinct pathways. Previous studies concerning the effect of FGF-2 on the PI hydrolysis in different cell types resulted in conflicting results [9–13]. Although the PI hydrolysis by FGF-2 has not been observed in some fibroblasts [11–13], PI-derived DG induced by FGF-2-activated PLC-γ1 could be converted to PA by DG kinase in our case. The PC hydrolysis by certain growth factors was also observed in some fibroblasts [14–17]. In the case of PC hydrolysis, PA could be formed through PLD action or the combination of phosphatidylcholine-specific phospholipase C (PC-PLC) and DG kinase actions. It has been shown, however, that membrane-bound DG kinase induces PA by preferentially phosphorylating PI-derived DG, but not PC-derived DG [22,23]. In the case of EGF and PDGF, it seems likely that they stimulate PLD rather than PC-PLC in some fibroblasts [14,15,17], even though Moscat's group has demonstrated that PC-derived DG may be sufficient to activate mitogenesis in fibroblasts without PA formation [16]. Recently, protein tyrosine phosphorylation has been proposed as a necessary step for PLD activation [24]. Moreover, the activation of PLD by the tyrosine kinase of v-Src was demonstrated [25]. It seems, therefore, likely that in our case, PA was induced via PLD by activating the tyrosine kinase of the FGF receptor rather than via PC-PLC. PA could be also formed through another pathway, de novo synthesis, involving glycerol kinase and acyltransferases. It has been reported that insulin induces PA through this de novo synthesis pathway [26]. Whether only one PA species among the PAs noted above (i.e. PI-derived, PC-derived and de novo synthesized) is in fact formed by FGF-2 and functions in our case, or whether several PAs are formed and function in conjunction with each other remains to be determined.

Consistent with previous reports [9,11], DG accumulation was observed in FGF-2-treated BHK cells in the present study. It was not determined, however, whether this DG accumulation was derived from the breakdown of PI or PC. Although it is well known that PI-derived DG can stimulate PKC, the precise function of PC-derived DG has remained unclear. Especially, whether this PC-derived DG can activate PKC or not was controversial [16,27,28]. Recent findings have raised the possibility that PC-derived DG may mediate mitogenic signals by stimulating novel types of PKCs, such as PKC ζ and ε [29,30].

Fukami et al. reported that the conversion of PA to DG is very low in PLD-treated Balb/c3T3 cells, and they suggested that PA by itself can induce DNA synthesis without DG formation [17]. In the present study, we could also observe only a slight accumulation of DG after PLD treatment. This observation supports the notion that in fact an elevation in PA levels upon FGF-2 and PLD treatment plays a critical role in triggering BHK cell proliferation.

PA could stimulate cell proliferation by a number of mechanisms [31]. For example, PA has been demonstrated to display an inhibitory effect on Ras GTPase activating protein (RasGAP) [32,33]. The interaction of

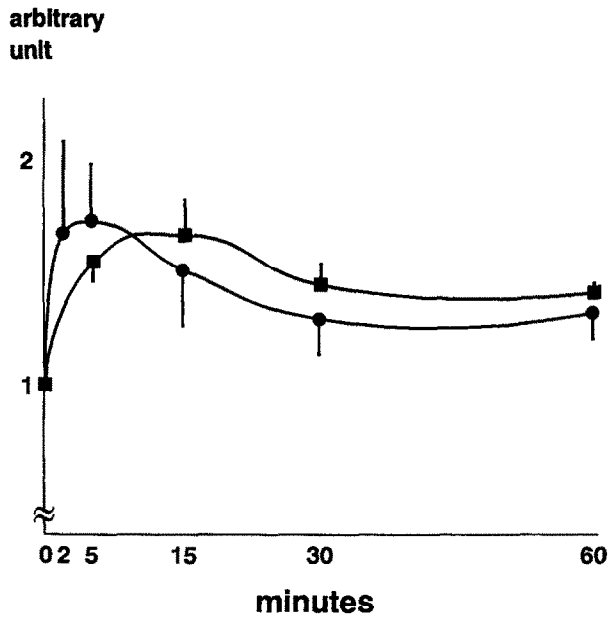

Fig. 3. Time course of accumulation of PA after the addition of FGF-2 (circles) and PLD (squares). Quiescent BHK cells were treated with FGF-2 (20 ng/ml) or PLD (5 U/ml) for the indicated time. Samples were processed, chromatographed and scanned as described in the text. The quantitated value of the untreated cells is expressed as 1.0 arbitrary unit. Data are mean standard deviation of three separate experiments.

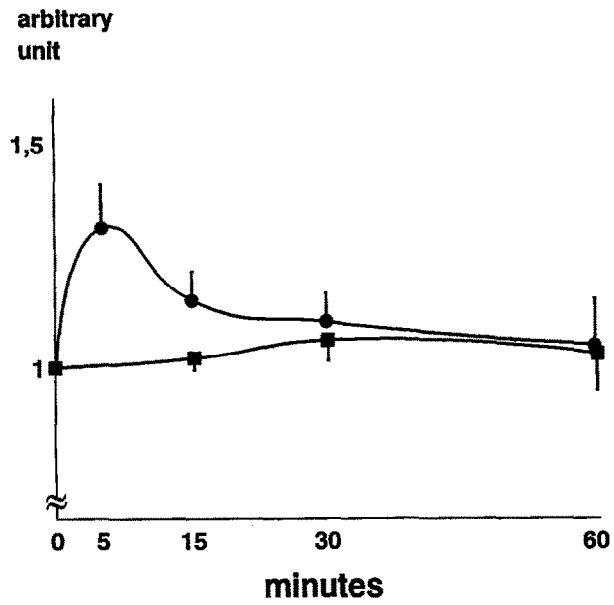

Fig. 4. Time course of accumulation of DG after the addition of FGF-2 (circles) and PLD (squares). Quiescent BHK cells were treated with FGF-2 (20 ng/ml) or PLD (5 U/ml) for the indicated time. Samples were processed, chromatographed and scanned as described in the text. The quantitated value of the untreated cells is expressed as 1.0 arbitrary unit. Data are mean standard deviation of three separate experiments.

PA with RasGAP may result in elevated cellular levels of the active GTP-bound form of Ras and thereby stimulate cell proliferation. At present, however, it is not understood whether or not PA induces cell growth through direct interaction with Ras.

It has also been reported that PA stimulates the phosphatidylinositol-4-phosphate kinase (PIPK) activity up to 20-fold in vitro, which may result in a faster replenishment of the PIP2 pool accessible for enzymatic breakdown by phosphatidylinositol-specific phospholipase C (PI-PLC) [34]. Moreover, enhanced breakdown of PIP2 was observed after addition of PA to various cell types and it has been proposed that this effect of PA is due to activation of PI-PLC, either directly or indirectly. Direct stimulation of PI-PLC by PA has been shown in in vitro studies [35].

The signals from the FGF receptor are initiated by FGF-2-induced activation of the intrinsic kinase activity of the receptor. This leads to tyrosine phosphorylation of some proteins as well as autophosphorylation of the receptor, which results in the association of PLC-γ1 and PI3K [7,8]. PLC-γ1 is a PIP2-specific phosphodiesterase and produces DG and IP3. PI3K is a lipid kinase that phosphorylates the D3 position of PI, phosphatidylinositol 4-phosphate (PIP) or PIP2. The real functions of these two enzymes are unknown at present [36–38]. Very recently, PLC-γ1 and PI3K were clearly recognized as the essential downstream mediators of the mitogenic signal mediated by the PDGF receptor [39]. If indeed, this mechanism turned out to be also operative in the case of the FGF receptor, FGF-2-induced PA may activate PIPK and PI-PLC as mentioned above, and this synergistic activation of PIPK and PI-PLC could then provide a powerful positive feedback signal for the amplification of FGF-2-induced mitogenic signals.

On the basis of our results, we suggest that PA plays an important role in FGF-2-induced BHK cell growth. We can not, however, exclude the possibility that FGF-2- or PLD-induced PA was degraded to lyso-PA, which may also play a critical role in the regulation of cell growth. Lyso-PA has been shown to be a strong mitogenic inducer [40]. This question remains to be resolved.

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