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Comparison of Bradykinin- and Platelet-Derived Growth Factor-Induced Phosphoinositide Turnover in NIH 3T3 Cells

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Abstract: Phosphoinositide turnover in response to platelet-derived growth factor, epidermal growth factor, and bradykinin was evaluated in NIH 3T3 cells. Platelet-derived growth factor and bradykinin induced a significant increase in incorporation of ^{32}P into phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2) in serum-starved NIH 3T3 cells. However, epidermal growth factor increased incorporation of ^{32}P into these phosphoinositides by only a small amount. Stimulation with platelet-derived growth factor, not bradykinin, caused a rapid elevation of PI and PIP kinase activities that were maximally activated within 10 min. The maximal levels of their elevation in cells with platelet-derived growth factor stimulation were 3.2-fold for PI kinase, and 2.1-fold for PIP kinase. Short term pretreatment of NIH 3T3 cells with phorbol 12-myristate 13-acetate, activator of protein kinase C, caused an approximately 60% decrease in platelet-derived growth factor-induced PI kinase activities, indicating the feedback regulation of phosphoinositide turnover by protein kinase C. These results suggest that although the enhancement of phosphoinositide turnover is a rapidly occurring response in platelet-derived growth factor- or bradykinin-stimulated NIH 3T3 cells, phosphoinositide kinases may be associated with initial signal transduction pathway relevant to platelet-derived growth factor but not to bradykinin.

Key words: phosphoinositide turnover, phosphatidylinositol kinase, phosphatidylinositol 4-monophosphate kinase, platelet-derived growth factor, bradykinin.

The increased turnover of phosphoinositides has been implicated in cellular responses to a wide variety of stimuli, including some growth factors and hormones such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), bradykinin, vasopressin, and bombesin in eukaryotic cells (Berridge and Irvine, 1984; Majerus *et al.*, 1990; Rana and Hokin, 1990). These external stimuli have been shown to exert their effects through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by the action of phospholipase C, which generates two second messengers, inositol 1,4, 5-trisphosphate and 1,2-diacylglycerol (Majerus *et al.*, 1990). The subsequent depletion of PIP_2 is restored by the synthesis from phosphatidylinositol through stepwise phosphorylations by phosphatidylinositol (PI) kinase and phosphatidylinositol 4-monophosphate (PIP) kinase. PI kinase has been shown to be physically associated with PDGF receptor (Coughlin *et al.*, 1989) or EGF receptor (Cochet *et al.*, 1991). Interestingly, it is also to be noted that activation of both PI kinase and phospholipase C requires mediation of cytosolic phosphatidylinositol transfer protein (Kauffmann-Zeh *et al.*, 1995).

Despite the simultaneous stimulation of phosphoinositide turnover by ligand occupation to their respective receptors, PDGF- and EGF-induced DNA syntheses are inhibited by bradykinin, which stimulates phosphoinositide turnover in several types of cells (McAllister *et al.*, 1993). In addition, bradykinin enhances phosphoinositide hydrolysis more efficiently than PDGF in v-Ki-ras transformed NIH 3T3 cells exhibiting the reduced level of phospholipase Cγ phosphorylation (Kumada *et al.*, 1994). Interestingly, phospholipase C was found to be physically associated with EGF receptor in NIH 3T3 cells overexpressing EGF receptor (Margolis *et al.*,

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1990). In NIH 3T3 cells overexpressing phospholipase C, PDGF enhanced phosphoinositide turnover, yielding 10-fold increase compared to the parental NIH 3T3 cells while bradykinin did not enhance the cycle (Renard *et al.*, 1992). Therefore, it is conceivable that although phosphoinositide turnover is stimulated by bradykinin, PDGF or EGF, its cycle through the action of them may be differently stimulated or modulated. In the present study, to obtain more insight into their respective regulation mechanisms of phosphoinositide turnover, we have undertaken experiments to evaluate the effects of EGF, PDGF, and bradykinin on phosphoinositide turnover by comparing their ability to induce PI and PIP kinases in NIH 3T3 cells. The present results demonstrate that activation of PI and/or PIP kinases is an initial response to PDGF stimulation but not to bradykinin stimulation in NIH 3T3 cells.

Materials and Methods

Materials

Dulbeccos modified eagles medium was purchased from GIBCO/BRL (New York, USA). EGF, PDGF, and bradykinin were from Sigma (St. Louis, USA). L-α-phosphatidyl 4-monophosphate, L-α-phosphatidylinositol 4, 5-diphosphate, L-α-phosphatidylcholine, L-α-phosphatidyl-L-serine, L-α-phosphatidylethanolamine, and phosphatidic acid were from Sigma. Silica Gel 60 thin-layer chromatography plates (0.25 mm layer thickness) were from Merck (Darmstadt, Germany). Carrier free Pi and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, USA).

Cell culture

NIH 3T3 cells were grown to a density of 0.5~1.0×10^5 cells per cm^2 on 35 mm dish (for extraction of phospholipids) or 100 mm dish (for preparation of particulate membrane fraction) in Dulbeccos modified eagles medium supplemented with 10% bovine calf serum, and were quiescent when shifted to the medium containing 0.5% bovine calf serum. Quiescent NIH 3T3 cells were washed twice with Dulbeccos modified eagles medium without phosphate and incubated with the medium containing carrier-free ^{32}Pi (20 μCi/ml) at 37℃ for 1.5 h to label the endogenous ATP pool. Then, EGF (100 ng/ml), PDGF (25 ng/ml), or bradykinin (1 μg/ml) was added for various times.

Phospholipid extraction and thin-layer chromatography

For measurement of the phosphorylation of phosphoinositides, phospholipid was extracted as described previously (Cohen *et al.*, 1971). Cells were scraped off with a rubber policeman and the cell suspension was mixed thoroughly with 400 μl of chloroform : methanol : c-HCl (100 : 200 : 4) for 1 min, and the mixture was allowed to stand for 10 min. The chloroform phase was separated by adding 200 μl each of chloroform and 2 M KCl, followed by centrifugation at 3,000×*g* for 2 min. The resulting lower phase was carefully collected, and the upper phase was rinsed once with 200 μl of chloroform. The combined chloroform phases were evaporated under a stream of nitrogen gas, and lipids were separated by thin-layer chromatography as described previously (Carney *et al.*, 1985). Briefly, half of the lipids extracted were spotted on silica gel 60 plate impregnated with 1% potassium oxalate and developed using two solvent systems, in an alkaline solvent of chloroform : methanol : 4 M NH_4OH (9 : 7 : 2) and in an acidic solvent of chloroform : methanol : acetic acid : distilled water (65 : 43 : 1 : 3). ^{32}Pi-labeled spots were detected by autoradiography on X-ray film and the individual lipid spots were scraped from the thin-layer chromatography plates and the radioactivity measured in a liquid scintillation counter.

Preparation of particulate membrane fraction

For measurement of PI kinase and PIP kinase activities, particulate membrane fractions were isolated by the methods of Ways *et al.* (1986). Cells were homogenized in homogenization buffer (20 mM Tris, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride). Cell homogenates were ultracentrifuged at 100,000 ×*g* for 60 min and the precipitates were extracted by sonication in homogenization buffer containing 0.3% (w/v) Triton X-100 followed by magnetic-stirring. The extracts were then centrifuged and the resulting supernatant fractions were used as a particulate membrane fraction.

Assay of PI kinase and PIP kinase

PI and PIP kinase activities were assayed as previously described by monitoring the transfer of γ-^{32}P from [γ-^{32}P]ATP to PI and PIP, respectively (Kato *et al.*, 1987). Assays were performed in a total volume of 50 μl in 20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 5 mM EGTA, 30 μg crude enzyme, and 50 nmol [γ-^{32}P]ATP (8.8×10^4 cpm/nmol). PI prepared by sonication or PIP dissolved in NP-40 (final concentration of 0.4%) was added to the respective reaction mixtures, reaching a final concentration of 250 μg/ml. The reactions were allowed to proceed for 10 min at 30℃ and were terminated by addition of 200 μl of methanol : 1 N HCl (1 : 1) and 200 μl of chloroform. All of the phosphoinositides extracted were chromatographed on potassium oxalate-impregnated Silica gel 60 plates in an

alkaline solvent as described above. The respective reaction products, PIP and PIP_2 were visualized by autoradiography, and the corresponding areas of thin-layer chromatography plate were scraped, and the radioactivities were measured in a liquid scintillation counter. The radioactivities counted were calculated as values of specific activities and presented as ratio of those of untreated control.

Results

Stimulation of phosphoinositide turnover by EGF, PDGF, and bradykinin

NIH 3T3 cells grown in complete medium were transferred to serum-free medium. These cells became quiescent and competent to the exposure of growth factors such as PDGF, which respond to a second set of growth factors that are required for their subsequent progression through the G1 and S phases of the cell cycle (Pledger *et al.*, 1977). The serum-starved NIH 3T3 cells were prelabeled with ^{32}Pi for 1.5 h prior to stimulation for 30 min with EGF, PDGF, or bradykinin and the lipids were separated by two solvent systems as described in Materials and Methods. Phosphatidic acid, phosphatidylserine, and PI migrated almost similarly in alkaline solvent, but separated distinctively in acidic solvent (Fig. 1). During the prelabeling period, incorporation of ^{32}Pi occurred mainly in phosphoinositides and phosphatidylcholine, slightly in phosphatidic acid and phosphatidylethanolamine but hardly in phosphatidylserine (Data not shown). Stimulation with PDGF or bradykinin caused a remarkable increase of ^{32}P incorporation into PI, PIP, and PIP_2 but stimulation with EGF led to only a slight increase (Fig. 1). The amounts of ^{32}P incorporation into phosphoinositides in cells stimulated by PDGF were similar to those in cells stimulated by bradykinin but much higher than those in cells stimulated by EGF, indicating that PDGF and bradykinin stimulate phosphoinositide turnover more actively than EGF. Poor response to EGF may be due to a low level of functional EGF receptors (3×10^3 receptors per cells) in NIH 3T3 cells (Di Fiore *et al.*, 1987). In this period, the accumulation of ^{32}Pi into phosphatidic acid, one of the major constituents of phosphoinositide turnover, was also increased by PDGF or bradykinin. However, the accumulation of ^{32}Pi into phosphatidylethanolamine, and phosphatidylserine was hardly affected by this stimulation. These results indicate that PDGF and bradykinin stimulated phosphoinositide turnover in NIH 3T3 cells, accompanying the increase of phosphatidylcholine.

Changes of PI and PIP kinase activities

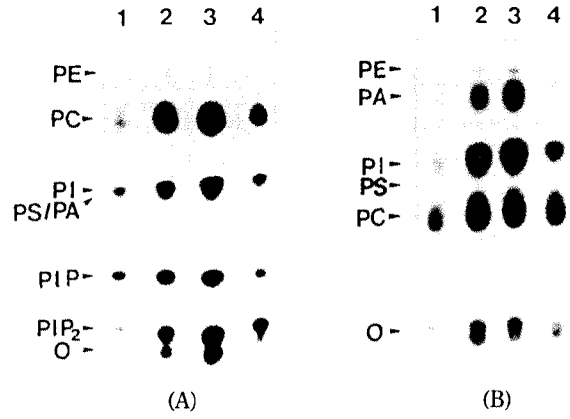

Fig. 1. Effects of PDGF, EGF, and bradykinin on phosphoinositide turnover. Serum-deprived quiescent NIH 3T3 cells were prelabeled with ^{32}Pi (20 μCi/ml) for 30 min at 37℃, followed by addition of phosphate-buffered saline (1), 1 μg/ml bradykinin (2), 25 ng/ml PDGF (3), or 100 ng/ml EGF (4) and then scraped out of the dishes. (A) The lipids were extracted from the cells and separated on 1% oxalate impregnated thin-layer plates using an alkaline solvent system. (B) A parallel experiment similar to A, except the lipid extracts were separated on thin-layer plates using an acidic solvent system. Positions of standard lipids are shown in the left. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidic acid (PA), origin of spots on thin-layer plates (O).

PDGF- and bradykinin-induced incorporation of ^{32}Pi into phosphoinositides suggested that PI and PIP kinase activities might be engaged in the response to these stimulators. Therefore, we compared the ability of EGF, PDGF, and bradykinin to stimulate these lipid kinase activities using particulate membrane fraction. Previous reports demonstrate that there are two types of PI kinases with distinct properties, which are inhibited or activated by non-ionic detergent such as NP-40 (Whitman *et al.*, 1987). Of these PI kinases, the detergent-inhibitable one is associated with the middle-T/pp60c-src and with PDGF stimulation. Thus, we measured PI kinase activities in the absence of NP-40 but PIP kinase activities in the presence of NP-40 using particulate membrane fraction at 48 h of serum deprivation where the specific activities were 137 pmol/min/mg and 15 pmol/min/mg, respectively (data not shown). The basal levels of PI kinase and PIP kinase activities were constant at 24 h, 48 h and 72 h after serum deprivation, indicating that this period is suitable for the measurement of the effect of bradykinin and platelet-derived growth factor on PI kinase and PIP kinase. Under these conditions, stimulation with PDGF caused a rapid induction of PI kinase activity, reaching a maximal value of 3.2-fold increase within 5 to 10 min after the addition and declining thereafter (Fig. 2). PI kinase was still elevated at 30 min after the exposure to PDGF. Similar activation patterns of PIP kinase were

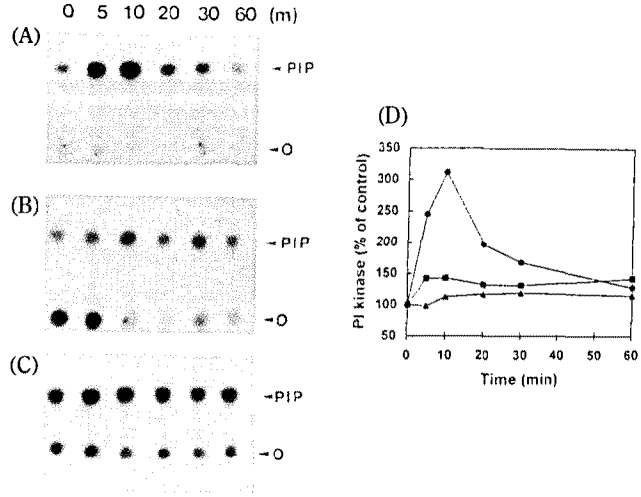

Fig. 2. Activation of PI kinase by PDGF. Quiescent NIH 3T3 cells were incubated with PDGF (A, —●—), EGF (B, —■—), or bradykinin (C, —▲—) as described in the legend of Fig. 1. (A, B, and C) The particulate membrane fractions were isolated, incubated with [γ-^{32}P]ATP and PI, and then autoradiography was performed. (D) The amounts of ^{32}P incorporated into PI were measured in a scintillation counter and each values are means of triplicate determination.

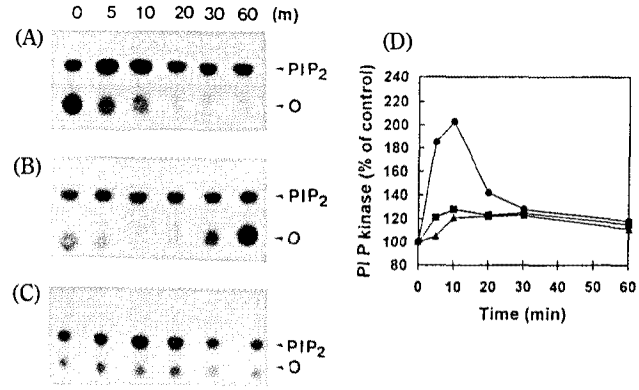

Fig. 3. Activation of PIP kinase by PDGF. Quiescent NIH 3T3 cells were incubated with PDGF (A, —●—), EGF (B, —■—), or bradykinin (C, —▲—) as described in the legend of Fig. 1. (A, B, and C) Autoradiograms showing PIP kinase activity are presented as radioactive spots of PIP_2. (D) The radioactivities of PIP_2 spots on thin-layer plates were counted and each values are means of triplicate determination.

obtained in PDGF-stimulated cells but the maximal activation levels of PIP kinase were somewhat lower than those of PI kinase, the extent of which was 2.1-fold. On the contrary, PI and PIP kinase activities were initially unchanged by EGF or bradykinin stimulation. These present findings indicate that the early increase in phosphoinositide turnover that occurs in quiescent NIH 3T3 cells in response to PDGF is due to a rapid activation of PI kinase and/or PIP kinase whereas the early increase in response to bradykinin may not be mediated by these lipid kinases. Rather, activation of phospholipase C may have a role in enhancement of phosphoinositide turnover by bradykinin. This consideration was supported by the observation that rapid hydrolysis of PIP_2 was initiated by bradykinin (Yano *et al.*, 1984).

Inhibition of PI kinase activity by phorbol 12-myristate 13-acetate pretreatment

It is well known that the activation of protein kinase C exerts a negative feedback regulation to phosphoinositide hydrolysis in most cell types, as a result of phosphorylation of a constituent in the signaling pathway of the receptor to phospholipase C. To examine whether the activation of protein kinase C affects growth factor-stimulated PI kinase in NIH 3T3 cells, the intact cells were pretreated for 10 min with various concentrations of phorbol 12-myristate 13-acetate, which elicits rapid elevation of protein kinase C, and then stimulated with PDGF, EGF, or bradykinin. As shown in Fig. 3, phorbol 12-myristate 13-acetate caused an inhibition of PDGF-stimulated PI kinase activity in a dose dependent manner, reaching a maximal inhibition above 40 nM. The inhibition of PI kinase by phorbol 12-myristate 13-acetate pretreatment was not observed in cells stimulated with bradykinin or EGF, each of which did not affect the activity of PI kinase. These present findings indicate that phosphoinositide turnover with PDGF stimulation is regulated through the inhibition of PI kinase by protein kinase C.

Discussion

PI and PIP kinases catalyze the sequential phosphorylation of phosphatidylinositol to yield PIP_2, the major substrate on which phospholipase C acts to produce the second messengers, inositol 1,4,5-trisphosphate and diacylglycerol. Activated PDGF, EGF and bradykinin receptors increase the formation of these second messengers and consequently increase the cytosolic Ca^{2+} and activate protein kinase C. The present study demonstrates that even though PDGF and bradykinin enhance the turnover of phosphoinositides in NIH 3T3 cells, PDGF stimulates the activities of PI and PIP kinases whereas bradykinin does not stimulate these kinase activities. These findings indicate that there are different mechanisms underlying the stimulation of phosphoinositide turnover between PDGF- and bradykinin-stimulated cells.

An increase in Ca^{2+} due to the release of Ca^{2+} from stores associated with the endoplasmic reticulum or to an external influx is an important intracellular mechanism that mediates the actions of a variety of hormones, neurotransmitters, and growth factors. PDGF, EGF,

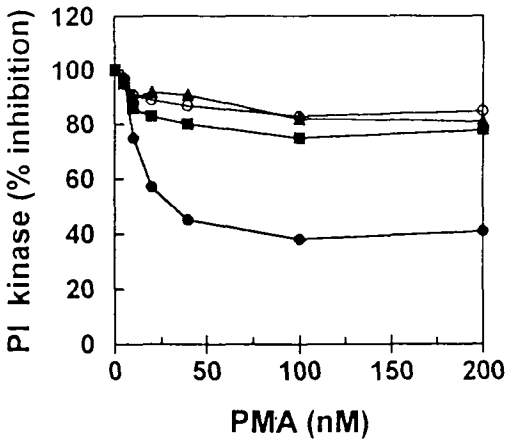

Fig. 4. Attenuation of PDGF-induced PI kinase activity by phorbol 12-myristate-13 acetate pretreatment. Quiescent cells were pre-incubated with phorbol 12-myristate 13-acetate (PMA) for 10 min, and then stimulated with phosphate buffered saline (-○-), PDGF (-●-), EGF (-■-), or bradykinin (-▲-) for 10 min. PI kinase activity was presented as a percentage of that of cells without pretreatment.

and bradykinin have been shown to elicit Ca^{2+} increase (Yano *et al.*, 1984; Pandiella *et al.*, 1989). However, the increase of Ca^{2+} in PDGF- and EGF-stimulated cells was transient whereas the increase in bradykinin-stimulated cells was sustained (Yano *et al.*, 1984; Pandiella *et al.*, 1989). Following bradykinin-stimulation, a biphasic increase in Ca^{2+} influx occurs, which consisted of an initial phase to allow rapid and transient increase of Ca^{2+}, and a second phase to produce sustained increase in Ca^{2+} influx (Yano *et al.*, 1984). Since PI kinase is inhibited by Ca^{2+} (Porter *et al.*, 1988), the sustained increase of Ca^{2+} by bradykinin stimulation may thus cause the inhibition of PI and PIP kinases. This might indicate why PI and PIP kinase activities were enhanced in PDGF-stimulated NIH 3T3 cells, but not in bradykinin-stimulated cells. Besides the sustained increase of Ca^{2+}, bradykinin also induces a prolonged increase in the content of 1,2-diacylglycerol in NIH-3T3 cells, whose increase was immediately mediated by hydrolysis of PIP_2 and then mediated by the hydrolysis of phosphatidylcholine (Fu *et al.*, 1992). Therefore, when cells were stimulated with bradykinin, the resulting increase of Ca^{2+} and 1,2-diacylglycerol induces an activation of protein kinase C, which may elicit inhibition of PI and PIP kinases. This suggestion was further evaluated in cells pretreated with phorbol 12-myristate 13-acetate, an activator of protein kinase C. The pretreatment with phorbol 12-myristate 13-acetate, leading to an activation of protein kinase C, produced the inhibition of PDGF-induced PI and PIP kinase activities in NIH 3T3 cells, illustrating the reason for the failure in inducing PI and PIP kinase activation in bradykinin-stimulated cells. Previously, bradykinin has been reported to induce the accumulation of phosphatidic acid (Owen and Boarder, 1991), which was then identified as an activator of PIP kinase *in vitro* (Moriz *et al.*, 1992). From these previous findings and our present results, it appears that even though the activation of PI and PIP kinase is not initially required for bradykinin-stimulated phosphoinositide turnover, the activation of PI and PIP kinase may be necessary for prolonged activation of phosphoinositide turnover by bradykinin.

Previous works have shown that EGF stimulates PI and PIP kinases (Walker and Pike, 1987; Cochet *et al.*, 1991) and that PDGF also enhances these activities (MacDonald *et al.*, 1987). The stimulation of PI and PIP kinases results from a direct interaction between these kinases and EGF or PDGF receptor (Thompson *et al.*, 1985; Coughlin *et al.*, 1989; Cochet *et al.*, 1991). In the present study, we detected an approximately 2- to 3-fold increase of PI kinase activity in PDGF-stimulated cells but only a slight (1.3-fold) increase of the activity in EGF-stimulated cells within 10 min. This observation may be due to a low level expression of functional EGF receptors in NIH 3T3 cells (Di Fiore *et al.*, 1987). It has also been reported that NIH 3T3 cells overexpressing EGF receptor induced a remarkable increase in phosphoinositide turnover, thereby enhancing DNA synthesis compared to their parental NIH 3T3 cells (Di Fiore *et al.*, 1987). Taken together, these previous findings and our present results thus clearly show the illustration for weak response to EGF in stimulating phosphoinositide turnover and in activating PI and PIP kinases.

As shown above, PDGF, EGF, and bradykinin can stimulate phosphoinositide turnover through several steps of its cycle. Our present findings suggest that activation of PI and PIP kinases might be a primary response to PDGF, rather than a secondary effect of phospholipase C-mediated depletion of the phosphoinositide pools whereas activation of phospholipase C could be an immediate response to bradykinin. This suggestion is supported by recent data demonstrating a rapid and transient thrombin stimulation of PIP_2 synthesis in platelets that occurs without activation of phospholipase C (Racaud-Sultan *et al.*, 1993). In addition, activation of PI and PIP kinases has also been suggested to be an initial response to PDGF stimulation (Lassing *et al.*, 1994). Therefore, from the previous suggestion and our present results, it appears that PI kinase and PIP kinase play an important role in the initial induction of phosphoinositide turnover in NIH 3T3 cells with PDGF stimulation, not with bradykinin stimulation.

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