Adhesion to Fibronectin Stimulates Inositol Lipid Synthesis and Enhances PDGF-induced Inositol Lipid Breakdown

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Abstract. The aim of these experiments was to investigate whether inositol lipids might mediate some of the effects of extracellular matrix (ECM) on cellular form and functions. The lipid phosphatidylinositol bisphosphate (PIP2) plays a role in cytoskeletal regulation while its hydrolysis products, diacylglycerol and inositol triphosphate, serve as second messengers. We therefore measured the effect of adhesion to fibronectin (FN) on PIP2 and its hydrolysis products, in the presence and absence of the soluble mitogen PDGF. PDGF induced a threefold increase in release of water-soluble inositol phosphates in C3H 10T1/2 fibroblasts when cells were attached to FN, but had little effect in suspended cells. Suppression of inositol phosphate release in unattached cells was not due to dysfunction of the PDGF receptor or failure to activate phospholipase C-γ; PDGF induced similar tyrosine phosphorylation of PLC-γ under both conditions. By contrast, the total mass of phosphatidylinositol bisphosphate (PIP2), the substrate for PLC-γ, was found to decrease by ~80% when cells were detached from their ECM attachments and placed in suspension in the absence of PDGF. PIP2 levels were restored when suspended cells were replated on FN, demonstrating that the effect was reversible. Furthermore, a dramatic increase in synthesis of PIP2 could be measured in cells within 2 min after reattachment to FN in the absence of PDGF. These results show that FN acts directly to stimulate PIP2 synthesis, and that it also enhances PIP2 hydrolysis in response to PDGF. The increase in PIP2 induced by adhesion may mediate some of the known effects of FN on cell shape and cytoskeletal organization, while regulation of inositol lipid hydrolysis may provide a means for integrating hormone- and ECM-dependent signaling pathways.

The inositol lipid pathway plays an important role in mediating the response of cells to a variety of hormones and growth factors. Hydrolysis of phosphatidylinositol bisphosphate (PIP2), by PIP2-specific phospholipase C (PLC) yields the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3). DAG activates protein kinase C (PKC), while IP3 triggers increased cytoplasmic calcium due to release from intracellular stores. In addition to its role as a substrate for PLC, PIP2 itself binds to the cytoskeletal proteins profilin (Lassing and Lindberg, 1985), gelsolin (Janmey and Stossel, 1987) and α-actinin (Fukami et al., 1992), causing release of actin by profilin and gelsolin, and enhanced binding of actin to α-actinin.

Recent studies demonstrate that extracellular matrix (ECM) molecules also activate intracellular chemical signal-
can be bypassed by using phorbol esters to activate PKC. These results suggest that inositol lipid hydrolysis might require both growth factors and adhesion to ECM. The observation that the increase in intracellular calcium normally triggered by PDGF in adherent fibroblasts was not observed in suspended cells (Tucker et al., 1990) supports this hypothesis.

These findings led us to undertake a direct analysis of inositol lipid turnover in response to FN. Our results show that adhesion to FN both increases the synthesis of PIP₂ in the absence of soluble factors, and enhances the breakdown of PIP₂ in response to PDGF.

Materials and Methods

Experimental System

C3H 10T1/2 fibroblasts were passaged in DME containing 10% FCS, penicillin (100 U/ml) and streptomycin (100 U/ml). For overnight incubations in suspension, cells were trypsinized and suspended in 1.5% methylcellulose (4,000 centipoise) in DME as previously described (Benecke et al., 1978). Cells were washed out of methylcellulose by dilution with PBS or medium followed by centrifugation at low speed. To maintain cells in suspension during experiments, they were placed on bacteriological dishes that were precoated with 0.19 mg/cm² poly-hydroxyethylmethacrylate (poly-HEMA) (Folkman and Moscona, 1978). To analyze the effects of attachment to FN, cells were plated onto bacteriological dishes which were coated with human plasma FN (Boehringer Mannheim Corp., Indianapolis, IN) at a saturation density of 2.5 µg/cm² as previously described (Ingber et al., 1990). Non-specific attachment sites were blocked using 1% BSA in PBS or BSA-free DME before use. Cells were stimulated with recombinant BB PDGF (Upstate Biotechnology, Saranac Lake, NY) at a concentration of 25 ng/ml.

Analysis of Water-soluble Inositol Phosphates

Cells were prelabeled to equilibrium with [3H]inositol by culturing cells for 3 d in inositol-free DME (Flow Laboratories, Irvine, CA) containing myo-(2-3H)inositol (4 µCi/ml; Amersham Corp., Arlington Heights, IL) and 10% dialyzed FCS. Then cells were trypsinized and plated onto either poly-HEMA or FN-coated dishes for 18 h in medium containing [3H]inositol and 0.5% FCS. LiCl was added to the medium (10 mM final concentration) 20 min before challenging the cells with PDGF. After 45 min in the presence of PDGF and LiCl, cells were washed and lysed in methanol/1 M HCl (1:1). The precipitate formed was removed by centrifugation and assayed for DNA content as described (Burton, 1956). The supernatant was then analyzed for inositol phosphates essentially as described (Lapetina and Siess, 1987). The supernatants were extracted with a 1:1 vol of chloroform, the aqueous fraction was removed to a new tube, dried under N₂, and reconstituted in 7 mM phosphate buffer (pH 7.0). An equal volume of chloroform was added. The organic phase was washed, reextracted with a solution of methanol/1 M HCl and an equal volume of chloroform was added. The organic phase was removed, and dried under a stream of N₂ gas. Lipids were separated by TLC using chloroform/methanol:4 M ammonium hydroxide (9:7:2). Radiolabeled lipids were detected by autoradiography and identified based on co-migration with radioactive PI, PIP, and PIP₂ standards. The labeling of the ATP was quantitated using a Molecular Dynamics Phosphoimage system (Molecular Dynamics, Inc., Sunnyvale, CA).

Immunoprecipitation and Immunoblotting

Cells were suspended in 1.5% methylcellulose or plated onto FN for 18 h in DME/0.5% FCS. After 18 h, the suspended cells were washed out of methylcellulose and plated on poly-HEMA or FN-coated dishes in DME/1% BSA. Cells were incubated at 37°C for 2 h before addition of PDGF. After 7 min, cells were washed with ice cold PBS, lysed in NP-40-containing immunoprecipitation buffer, and immunoprecipitated with a specific antibody to PLC-γ (kindly provided by Tony Pawson) (Decker et al., 1990), according to published methods (Morrison et al., 1990). Immuno-precipitated proteins were separated on a reducing 7.5% SDS-PAGE minigel, transferred electrophoretically to nitrocellulose, and probed with a mAb to phosphotyrosine (gift of Tom Roberts). Anti-phosphotyrosine reactive bands were visualized using 125I-labeled sheep anti-mouse immunoglobulin (Amersham Corp.) followed by autoradiography.

Determination of the Total Mass of Unlabeled PIP₂

In experiments investigating the effect of cell detachment on PIP₂ mass, confluent cells were incubated in DME/0.5% FCS for 18 h. Cells were then trypsinized and plated on dishes coated with FN or poly-HHEMA in DME/1% BSA. To analyze the effects of cell attachment to FN, cells were either suspended in methylcellulose or plated on FN for 18 h in DME/0.5% FCS. Suspended cells were washed out of methylcellulose and replated onto either poly-HHEMA or FN for 2 h in 0.5% FCS. Cells adherent to FN were also incubated for an additional 2 h. To analyze the effects of cell detachment, confluent monolayers were incubated overnight in DME/0.5% FCS, trypsinized and resuspended in DME/1% BSA, and plated on dishes coated with FN or poly-HHEMA. After experimental manipulation, cells were washed in cold PBS, lysed were extracted, and PIP₂ was quantitated using a commercially available kit (Amersham Corp.) based on an adaptation of the method of Chliveris (Chilvers et al., 1991). Briefly, the extracted lipids were hydrolyzed in 1 M KOH and the released PIP₂ was quantitated using a competitive binding assay. The total mass represented by the amount of PIP₂ released was calculated based on the formula provided by the manufacturer. In this experiment, cell number was determined in duplicate samples using an assay based on quantitation of cellular acid phosphatase (Connolly et al., 1986).

Analysis of Inositol Lipid Synthesis

Cells were maintained for 18 h in 1.5% methylcellulose culture in DME containing 10% FCS. The suspended cells were collected and transferred to dishes coated with poly-HHEMA in P02-free DME (Flow Laboratories) containing 1% BSA and 20 µCi/ml 32P04. After 2 h of labeling, cells were plated in dishes coated with either FN or poly-HHEMA. In the experiment depicted in Fig. 3, cells were plated onto appropriately coated wells of a 6-well dish and centrifuged briefly at low speed to synchronize attachment. At the indicated times, cells were lysed and extracts extracted as described (Lapetina and Siess, 1987). In brief, cells were washed with ice cold PBS, lysed in a 1:1 solution of methanol/1 M HCl and an equal volume of chloroform was added. The organic phase was removed, reextracted with a solution of 100 mM EDTA/methanol (0.9:1) and dried under a stream of N₂ gas. Lipids were separated by TLC using chloroform/methanol:4 M ammonium hydroxide (9:7:2). Radiolabeled lipids were detected by autoradiography and identified based on co-migration with radioactive PI, PIP, and PIP₂ standards (Amersham Corp.). Lipid spots were scraped from the TLC plates and radioactivity was quantitated by liquid scintillation counting.

For the ATP specific activity determination, cellular ATP levels were measured using a commercially available kit (Sigma Immunochromatics, St. Louis, MO). Cells were lysed in the buffer provided at 200,000 per ml, and 100 µl of a 1:4 dilution was loaded into the sample chamber of a Wallac model 1251 luminometer. The luciferase/luciferin mixture (100 µl of a 1:25 dilution of the solution provided) was injected, and the light emission measured from 2 to 32 s after injection. ATP levels were determined by comparison with a calibration curve.

To assess the incorporation of radiolabel into ATP, cells were labeled with 32Pi and as described above, and protein-free extracts prepared by lysis in 2% perchloric acid. Lysates were chilled for 20 min on ice, then centrifuged to remove the precipitated protein. Supernatants were brought to pH 8 by addition of 1 M K₂CO₃, and 5 ml samples spotted on polyethyleneimine-cellulose TLC plates (Baker Co., Sanford, ME) that had been prerun in distilled water. The plates were developed in 1 M LiCl/1 M formic acid as described (Tones et al., 1988). 32Pi and 32P04-ATP were run as standards. The labeling of the ATP was quantitated using a Molecular Dynamics Phosphoimage system (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Adhesion Enhances PDGF-stimulated PIP₂ Hydrolysis

We first investigated whether regulation of inositol lipid hydrolysis by a soluble agonist depends upon adhesion to ECM. The ability of PDGF to induce release of water-soluble inositol phosphates in C3H 10T1/2 fibroblasts that were either adherent to FN or in suspension was therefore assayed. Cells were grown to confluence in medium with [3H]inositol, then either plated on FN or put in suspension culture using dishes coated with the nonadhesive polymer, poly-hydroxyethylmethacrylate (poly-HHEMA) as described.
studies, addition of PDGF induced a threefold increase in inositol phosphate release in adherent cells (Fig. 1). In contrast, PIP2 hydrolysis appeared to be inhibited in suspended PDGF stimulation (Fig. 1). The level of water-soluble inositol lipids breakdown. These results demonstrate that ceils, indicating that adhesion to FN had little direct effect on inositol phosphates released in adherent cells in the absence of cells, since much less IP release could be measured after PDGF was added in the presence of LiCl, and after 45 min the inositol phosphates were analyzed. Inositol phosphate release was normalized to cell number based on DNA content and represents the sum of IP, IP3, and IP2. Units for inositol phosphate release are cpm per 10^3 cells. The experiment was performed in triplicate, and similar results were achieved in two experiments. Values are means ± standard deviation.

(Folkman and Moscona, 1978). As expected from previous studies, addition of PDGF induced a threefold increase in inositol phosphate release in adherent cells (Fig. 1). In contrast, PIP2 hydrolysis appeared to be inhibited in suspended cells, since much less IP release could be measured after PDGF stimulation (Fig. 1). The level of water-soluble inositol phosphates released in adherent cells in the absence of PDGF was only slightly elevated over that in unattached cells, indicating that adhesion to FN had little direct effect on inositol lipid breakdown. These results demonstrate that anchorage-dependent fibroblasts must adhere to ECM to hydrolyze maximal amounts of PIP2 in response to PDGF.

A possible explanation for the diminished responsiveness in cells lacking contact with ECM is that either the PDGF receptor or PLC-γ, the enzyme that hydrolyzes PIP2, could be absent or could fail to be activated in response to PDGF. The possibility that the PDGF receptor was dysfunctional in suspended cells was unlikely since analysis of whole cell extracts by immunoblotting with anti-phosphotyrosine antibody showed that PDGF induced a similar protein phosphorylation pattern in both suspended and adherent cells (not shown). To test whether PLC-γ activation was inhibited in suspended cells, we measured the phosphorylation of PLC-γ on tyrosine, a modification that has been previously shown to be sufficient for enzyme activation (Kim et al., 1991; Padeh et al., 1991). Immunoprecipitation of PLC-γ, followed by immunoblotting with anti-phosphotyrosine antibodies, revealed that PDGF induced phosphorylation of PLC-γ equally well in suspended and adherent cells (Fig. 2). A protein band that migrated with an apparent molecular mass of ~145 kD, the expected molecular weight of PLC-γ, was recognized by the anti-phosphotyrosine antibody in immunoprecipitates from cells stimulated with PDGF. A second tyrosine-phosphorylated band appeared at ~185 kD, the expected molecular weight of the PDGF receptor. This finding is consistent with previous studies that demonstrated coprecipitation of the PDGF receptor with phosphorylated PLC-γ (Morrison et al., 1989) and supports our contention that the PDGF receptor remains functional in both adherent and suspended cells. Taken together, these results indicate that both the PDGF receptor and PLC-γ remain present and responsive to PDGF in suspended cells. The data therefore suggest that adhesion to FN must alter inositol lipid hydrolysis by acting at some point other than PLC activation.

**PIP2 Mass Decreases in Unattached Cells**

We then examined whether FN had any direct effects on the inositol lipid pathway in the absence of PDGF. The effect of adhesion to FN on the levels of cellular PIP2, the substrate for PLC-γ, was determined using a commercially available assay for total PIP2 mass that does not require metabolic labeling, and therefore avoids any possible labeling artifacts (Chilvers et al., 1991). The assay involves hydrolysis of PIP2 to IP3, and measurement of the IP3 by a competition binding assay similar to a radioimmunoassay. Fig. 3 b shows that the total mass of PIP2 rapidly decreased after cells were detached from the substratum and held in suspension. Replating suspended cells onto FN in serum- and growth factor-free medium resulted in a fivefold increase in the total amount of cell-associated PIP2, that occurred over several hours (Fig. 3 a). Cells that had been labeled to equilibrium with [3H]inositol demonstrated a similar decrease in PIP2 upon detachment, and a similar increase upon reattachment (data not shown). These experiments also revealed that the total [3H]inositol incorporated into lipid, which is predominantly PI, was unaffected by adhesion, (0.76 and 0.78 cpm/cell in suspended and adherent cells, respectively). Thus, the changes measured in PIP2 are not the result of general changes in inositol lipid metabolism. These results demonstrate that the quantity of cellular PIP2 is specifically
and reversibly regulated by adhesion to FN in the absence of growth factors.

Addition of PDGF to cells either in suspension or at the time of plating on FN did not result in any change in the mass of PIP2 4 h later (data not shown). This result shows that PDGF-induced changes in the rate of PIP2 formation are sufficient to replace any PIP2 hydrolyzed in response to PDGF, but that FN is the primary determinant of PIP2 mass. PDGF was observed to cause a decrease in the mass of PIP2 15 min after addition to adherent cells, but no such decrease could be detected in cells in suspension (not shown). These results are consistent with previous work showing that PDGF induces a transient decrease in PIP2 levels in adherent cells followed by a return to initial levels at later times (Rana and Hokin, 1990), and also support the data suggesting that little PIP2 breakdown occurs in suspended cells.

**Attachment to FN Stimulates a Rapid Increase in PIP2 Synthesis**

FN could affect the level of PIP2 via modulation of its synthesis.
Table I. Analysis of 32P-ATP Pools

| Cells            | Radiolabel in ATP* (% control) | ATP levels† (% control) |
|------------------|-------------------------------|-------------------------|
| Suspended        | 100                           | 100                     |
| On FN 20 min     | 112 ± 15                      | 97 ± 11                 |

* Cells were radiolabeled with 32PO4 for 2 h in suspension as described for Fig. 4, then either plated on FN or maintained in suspension as in Fig. 4. After 20 min, the cells were lysed, and the amount of radioactivity in ATP determined. The samples were done in duplicate, and two experiments yielded identical results. Data are shown as means ± range.
† Suspended cells were plated on FN or maintained in suspension as in Fig. 4. After 20 min, they were lysed, and cellular ATP levels assayed. Samples were done in quadruplicate, and data are shown as means ± standard deviation.

Discussion

To summarize, our data show that adhesion to FN both induces PIP2 synthesis and modulates PIP2 hydrolysis in response to PDGF. This second point confirms previous work showing that PDGF fails to activate PKC (Schwartz and Lechene, 1992) or trigger calcium release in suspended cells (Tucker et al., 1990).

The change in PIP2 synthesis induced by cell binding to FN was both rapid (within minutes) and dramatic, representing a three- to fivefold increase when measured by either radiolabeling techniques or nonradioactive methods. ~98% of the PIP2 synthesized in response to FN was confirmed by HPLC to be the classical isomer, PI(4,5)P2, with <2% being PI(3,4)P2 (Kapeller, R., H. McNamme, D. Inger, L. Cantley, and M. Schwartz, unpublished data), a novel lipid which is not a substrate for PLC (Serunian et al., 1991). Inositol lipid measurements also revealed a decrease in the amount of 32PO4-labeled PIP2 minutes after cell binding to FN-coated dishes. Taken together, these results suggest that adhesion to FN acts specifically to increase the activity of the PIP 5-kinase, the enzyme that phosphorylates 4-PIP to 4,5-PIP2. The data are inconsistent with a general increase in inositol lipid turnover or in overall cellular lipid metabolism.

One possible model that is consistent with our data is that FN regulates inositol lipid breakdown in response to soluble mitogens by controlling the amount of PIP2 available for hydrolysis. In this model, PDGF does not stimulate maximal release of the second messengers DAG and IP3 in nonadherent cells because the level of PIP2 is low. Data indicating that PIP2 synthesis can be rate limiting for inositol lipid hydrolysis has been obtained in several systems (Urumow and Wieland, 1986; Taylor et al., 1984; Chahwala et al., 1987). Furthermore, given the magnitude of the changes in PIP2 mass due to adhesion, and the fact that cells usually contain significant pools of PI lipids that are not available for hydrolysis by hormone-sensitive PLC (Inokuchi and Imboden, 1990), it is not unreasonable that the change in total cellular PIP2 could represent a complete loss of hormone-sensitive PIP2. Under those conditions, the reduction in PIP2 could be sufficient to explain the greatly reduced hydrolysis of PIP2 in response to PDGF in suspended cells. However, whether or not this model is the correct one remains to be determined. Additional explanations for the failure of PLC-γ to hydrolyze PIP2, such as incorrect localization or compartmentalization of PLC-γ in the cell, cannot be excluded by the current data.

Whatever the mechanism, the result that both FN and a soluble agonist are required for maximal PIP2 hydrolysis may provide, at least in part, a molecular basis for the observation that optimal growth of normal cells requires both anchorage and soluble mitogens. Additionally, there are a number of cell systems in which cell differentiation or activation is modulated by adhesion to ECM in combination with soluble hormones or cytokines (Nathan et al., 1989; Jhabvala and Bixby, 1990; Emerman et al., 1977). In some cases, the requirement for ECM can be filled by exogenous activation of PKC using phorbol esters (Nathan et al., 1989; Jhabvala and Bixby, 1990), consistent with an effect of adhesion on inositol lipid hydrolysis. Thus, the effect observed here could be relevant to systems where ECM regulates cell growth, differentiation or other functions.

It also seems likely that induction of PIP2 synthesis by FN may mediate some of the effects of adhesion on the actin cytoskeleton. PIP2 binds to and regulates the activity of gelsolin, profilin and α-actinin (Fukami et al., 1992; review see Stossel, 1989). Binding of PIP2 to gelsolin induces dissociation of gelsolin from actin, leaving free barbed actin filament ends. PIP2 binding to profilin leads to dissociation of the profilactin complex and an increase in free, polymerization-competent actin. Binding of PIP2 to α-actinin leads to enhanced actin binding, and may be of particular relevance, since α-actinin appears to serve as a link between actin and the cytoplasmic domain of the integrin β1 chain (Otey et al., 1990; Pavalko and Burridge, 1991). Increased synthesis of PIP2 would therefore be predicted to increase actin polymerization and possibly promote the association of actin with integrins, consistent with the known effects of cell adhesion to FN.

Finally, it has generally been thought that turnover of inositol lipids is regulated primarily by agents that promote their hydrolysis, whereas their synthesis is constitutive, or...
occurs after depletion by hydrolysis. Our results show that synthesis and breakdown of PI(4,5)P_2 can be regulated by distinct extracellular agents. A complete understanding of the inositol lipid cycle and its role in regulating cell functions will therefore require further investigation into the control of inositol lipid synthesis by integrins.

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