Inhibition of Phospholipase D Activity by Fodrin

AN ACTIVE ROLE FOR THE CYTOSKELETON*

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Phospholipase D (PLD) is a major enzyme implicated in important cellular processes such as secretion and proliferation. The knowledge of its regulation is essential to understand the control of these phenomena. Several proteins activating PLD have been described in the last years. In this report, we chromatographed bovine brain cytosolic proteins to identify fodrin, the non-erythroid spectrin, as the first described inhibitor of PLD. A cytosolic fraction with an inhibitory effect on PLD activity loses its capacity after immunoprecipitation of fodrin. Moreover, at 1 mM, purified fodrin blocks fully and quickly PLD activity, whatever the stimuli used. In contrast, fodrin has no effect on adenylate cyclase activity. Fodrin-analogous proteins like dimeric or tetrameric erythroid spectrin have the same inhibitory effect on PLD, at higher concentrations. Other cytoskeletal proteins, actin and vimentin, are inefficient on PLD inhibition. The mechanisms implicated in PLD modulation such as post-translational modifications of fodrin and the role of small G-proteins on the cytoskeleton regulation are discussed. In conclusion, this study reveals that fodrin is involved in the control of PLD activity, suggesting that the cytoskeleton could have an active role in control of secretion and proliferation.

Phospholipase D (PLD) activity was shown to occur in a large number of intact cell types after triggering of receptors by agonists, including hormones, neurotransmitters, and growth factors. The involvement and the importance of PLD activity has been demonstrated in major physiological processes such as proliferation and secretion (reviewed in Refs. 1–3). In mammalian cells, phosphatidylcholine (PC), the most abundant charged phospholipid has been shown to be biologically active (reviewed in Ref. 3), it possesses fusogenic properties (4), and it is involved in DNA synthesis and proliferation of fibroblasts (5, 6). It is supposed to be a second messenger in secretory processes in several cell types, including pancreatic islets and adrenal glomerula cells (reviewed in Ref. 1). PLD in mammalian cells, although not yet shown, seems to be tightly bound to plasma membranes. Due to the loss of enzyme activity during membrane protein solubilization, purification of PLD from mammalian cells has been unsuccessful for almost two decades. Over the past 2 years, proteins with PLD activity were partially purified with mild detergents (7, 8), and ARF-stimulated PLD cDNA has been cloned recently (9).

Regulation of PLD activity is not yet fully understood. PLD activation pathways include stimulation by Ca2+, activation of PLC, and of different kinases (protein kinases C and tyrosine kinases) leading to phosphorylation cascades. Moreover, a positive regulation of PLD activity by G-proteins has been widely studied in different cell types but only indirect demonstration of heterotrimeric G-protein involvement in the enzyme activation has been made so far (reviewed in Ref. 1). In contrast, it has been clearly demonstrated that three small proteins with GTPase activity are stimulators of PLD activity: ARF (10, 11), rhoA (12–14), and p21ras in v-Src-transformed cells (15, 16). Moreover, another cytosolic factor, identified as PKCα, was recently demonstrated to be involved in the stimulation of PLD activity (17, 18).

Thus, in mammalian cells, regulation of this enzyme seems to be under the control of neighboring elements. The negative regulation of PLD has been reported to be under the control of cellular cytosol factor(s) not yet identified. During the initial step of purification of cytosolic PLD activators, a major increase in the total stimulating PLD activity was observed, suggesting the removal of an inhibitor. In a previous work, we have reported the presence in bovine brain cytosol of a high molecular mass factor, either a protein or a complex of proteins, which negatively regulates PLD activity (19). In this report, we identify this inhibitor as a single protein, fodrin, the non-erythroid spectrin. This protein is an actin-binding protein participating in the cytoskeleton. We also investigate the effect of fodrin on the enzyme activity.

EXPERIMENTAL PROCEDURES

Materials

RPMI 1640, medium 199, and supplements were purchased from Life Technologies, Inc.; fetal bovine serum was from Dutcher. Streptolysin O was obtained from Murex, nucleotides and calmodulin from Boehringer Mannheim, and calpain I was purchased from Calbiochem. Horseradish peroxidase-labeled antibody against rabbit IgG and ECL Western blotting detection reagents were obtained from Amersham Corp. as well as [methyl-3H]choline chloride (75–85 Ci/mmol). l-myo-[2-3H]inositol (20 Ci/mmol) and [5,6,8,9,11,12,14,15-3H]arachidonic acid (200 Ci/mmol)
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were from DuPont NEN. Nitrocellulose membranes and Bio-Rex 70 cation exchange column (sodium form) were purchased from Bio-Rad. Scintillant liquid, OptiPhase “HiSafe” 3, was from EG&G Wallac. Chromatography supports (except heparin-agarose beads) and columns for fast flow liquid chromatography were purchased from Pharmacia-LKB. All other reagents were from Sigma.

Specific polyclonal antiserum against pig brain fodrin and pure lens vitrein were kindly given by Louise Anne Pradel, Institut de Biologie Physico-Chimique, Paris, and Karima Djabali, CNRS, Collège de France, Paris, France, respectively.

HL-60 cells given by Dr T. Breitman, NCI, Bethesda, MD, were cultured in suspension in RPMI 1640 medium as described (20). For labeling, medium 199 was chosen because of its low concentration in choline. Cells were harvested and resuspended at 0.5 × 10^6 cells/ml in the medium containing 10% heat-inactivated fetal bovine serum, and 0.5 μCi/ml [methyl-3H]choline chloride was added. Cells were cultured for 72 h at 37°C in a humidified incubator containing 5% CO2 to reach isotopic equilibrium (21).

Methods

Cell Permeabilization with Streptolysin O and PLD Activity Measurement

After labeling, cells were washed three times in buffer A, pH 6.8, made of 137 mM NaCl, 2.7 mM KCl, and 20 mM Pipes and permeabilized at 4 × 10^6 cells/ml in buffer A with 0.4 unit/ml streptolysin O.

PLD activity can be studied by measurement of both products, chol- oline or PA. PLD possesses a specific transphosphatidylation activity of PtdEtOH is highly specific of PLD in comparison with choline measurement. Briefly, as already mentioned choline-containing lipids were measured by liquid chromatography as described by Martin (23).

Vesicles containing various concentration of PC with or without 125I-PIP2 were made according to Brown and Sternweis (29). Vesicle formation, as reported previously (21) because of the rapidity of this measurement. We have previously checked that the inhibitory cytotoxic factor provoked a decrease in the formation of both products of PC metabolites as described by Martin (23).

Preparation of Bovine Brain Cytosol

All steps were carried out at 4°C.

Gray tissue of one bovine brain (200 g) was cut into small pieces and put in 1 liter of a buffer consisting of 10 mM Tris-HCl, buffered at pH 7.6, containing 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 2% glycerol, protease inhibitors as above plus 1 μg/ml diisopropyl fluorophosphate (buffer B). Tissue was then homogenized in a Waring blender (10 × 1 min). The homogenate was centrifuged for 14 h at 16,000 × g, and fractionation was undertaken. After centrifugation, cytosolic proteins in the supernatant were precipitated for 30 min with 80% (NH4)2SO4 by addition of solid salt and collected by centrifugation at 15,000 × g for 30 min. Precipitated proteins were dissolved in 100 ml of buffer B.

Chromatography on DEAE-Sepharose Fast Flow—After extensive dialysis against buffer B, soluble proteins (100 ml) were applied on colohchromatography on DEAE-Sepharose (5 × 26 cm), an anion exchange gel equilibrated with buffer B. After extensive washing, retained proteins were eluted at 0.8 ml/min with a linear gradient of 1 liter of buffer A versus 1 liter of the same buffer containing 1 mM NaCl. Fractions of 20 ml were collected.

Chromatography on Immobilized Heparin—The peak containing the PLD inhibitory activity was pooled and dialyzed against buffer B and loaded onto a heparin-agarose column (2.5 × 20 cm) equilibrated with the same buffer. After buffer washing to remove unretained material, proteins with affinity for heparin were eluted at 30 ml/h with a linear NaCl gradient (0–1, 1 liter), 8 ml fractions were collected.

Chromatography on a Hi-Trap Q Column—The active fractions were pooled and dialyzed against the same buffer as for DEAE. Proteins of the supernatant were chromatographed on a 5 ml Hi-Trap Q column (Pharmacia Biotech Inc.), an anion exchange column, equilibrated with the same buffer, and eluted at 1 ml/min with a linear NaCl gradient (0–500 mM, 200 ml). Fractions of 2.5 ml were collected.

Chromatography on a Phenyl-Superose Column—Fractions containing the activity were pooled, dialyzed against buffer B, except that glycerol was omitted, adjusted to 0.8 M (NH4)2SO4 with 4 M concen-

trated solution, and applied to a phenyl-Superose high performance column (1.6 × 8 cm) equilibrated in a Tris buffer with the same final salt concentration as the sample. After washing, retained proteins were eluted with a decreasing linear salt gradient, and 2.5-ml fractions were collected and desalted on PD 10 column (Pharmacia).

Filtration Chromatography—The peak containing PLD inhibitory activity after chromatography on phenyl-Sepharose was eluted in the absence of ammonium sulfate salt. The active peak was pooled, concentrated, and dialyzed against a 10 mM Tris-HCl buffer, pH 7.6, and 0.5 ml was loaded on a Superdex 200 column (1.6 × 30 cm, Pharmacia) and eluted at a flow rate of 0.3 ml. Fractions of 0.5 ml were collected.

After each step of chromatography, each fraction was tested for adsorbance at 280 nm, and 50 μl of each eluted fraction was tested for its ability to inhibit PLD activity. The rapidity in the inhibitor purification was essential as we observed that this factor was not stable although a large range of protease inhibitors were always present in buffers.

Preparation of Brain Fodrin

Fodrin was extracted from bovine brain membranes at low ionic strength with dithiothreitol (1 mM) and purified according to the molecular mass on a gel filtration column (Bio-Gel A-5m 100–200 mesh) followed by chromatography on a weak anion exchange column (DEAE-Sepharose) according to Davis and Bennett (28).

Fodrin Treatment with Phospholipid Vesicles

Vesicles containing various concentrations of PC with or without 125 μM PIP2 were made according to Brown and Sternweis (29). Vesicle aliquots of 25 μl were incubated with 25 μl of either buffer, pH 7.6, made of 20 mM Tris and protease inhibitors as above or fodrin (10 nM in the same buffer) in the presence of MgCl2 (2 mM) Mg-ATP (2 mM) and Ca2+ (10 μM). After 30 min at room temperature, PLD activity was measured in the absence and in the presence of the vesicles with or without fodrin in permeabilized cells as described above.
Preparation of Erythroid Spectrin

Spectrin was extracted by incubating white erythrocyte membranes in low ionic strength buffer (0.3 mM Tris, pH 8, 0.1 mM 4-(2-aminooethyl)benzenesulfonyl fluoride (AEBSF), 0.1 mM EDTA, 0.1 mM β-mercaptoethanol) either for 30 min at 37°C to obtain spectrin dimers or overnight at 4°C to obtain spectrin tetramers. Spectrin extracts, separated from membrane residues by centrifugation, were dialyzed at 4°C against 150 mM NaCl, 25 mM Tris, pH 7.4, containing β-mercaptoethanol and anti-proteases: AEBSF, aprotinin, and leupeptin).

Protein Estimation

Protein content in the different fractions from bovine brain cytosol was estimated by light absorbance at 280 nm for column elution and using the technique described by Bradford (30) when a precise amount of pure proteins was required.

Fraction Analysis and Immunoblotting

30 μl of each fraction eluted after chromatography were mixed with 15 μl of SDS-PAGE sample buffer (three times) and boiled for 2 min. Proteins were separated by SDS-PAGE (31). Proteins were transferred onto nitrocellulose membranes by semidry Western blotting according to Burnette (32) except that the second antibody was peroxidase-linked anti-rabbit IgG and the blot was developed using ECL reagents.

Immunoprecipitation Assay

Cytosolic fractions (1 ml) from DEAE chromatography containing PLD inhibitory activity were preclared with protein A-Sepharose beads and then incubated at 4°C for 2 h, on a rotating wheel, with affinity-purified fodrin antibodies (5 μg/1.4 mg of proteins) or with the same amount of rabbit IgG. The incubate was then adsorbed onto protein A-Sepharose beads. A sequential run of immunoprecipitation was performed to fully deplete fodrin from fractions. After centrifugation of beads, the cytosolic fractions were analyzed for their effect on PLD activity by and immunoblotting for their content in fodrin.

RESULTS

Identification of the Inhibitor of PLD Activity—We took advantage of cell treatment with streptolysin O, which produces major holes in the plasma membrane, to isolate and identify a cytosolic inhibitor for PLD. To detect any inhibition, PLD activity was measured in whole permeabilized cells, i.e. in the presence of their cytosol which contains PLD activators.

Purification of the inhibitor was attempted by precipitation of cytosolic proteins by 80% ammonium sulfate followed by chromatography on a weak anion exchange resin, DEAE-Sepharose fast flow. One peak with inhibitory activity was eluted and corresponding fractions were pooled, concentrated, and further purified on immobilized heparin followed by chromatography on a strong anion resin, Hi-Trap Q, and on phenyl-Superose. Separation of proteins according to their molecular weight was performed by gel filtration on a Superdex 200 column. Fractions containing the inhibitory activity for PLD were eluted in one peak in the void volume, indicating a mass over 700 kDa for the inhibitory factor. Protein analysis by SDS-PAGE showed that fractions with inhibitory activity contained several proteins with molecular masses ranging from 40 to 260 kDa. However, only high molecular mass proteins, particularly a doublet at around 220 kDa and a band at 150 kDa, fitted with the peak of inhibition (Fig. 1, A and B). We have previously eliminated that this factor was the adaptin or the COP complex as none of these proteins were found in the inhibitory fraction (19). Considering that PLD is located at the plasma membrane level, near the cytoskeleton, we suspected that the inhibitor could belong to this cellular structure and that the doublet could be the non-erythroid spectrin, fodrin, which shares these characteristics. Using specific antibodies against fodrin, it was confirmed by immunoblot analysis that the doublet in fractions containing the inhibitory effect on PLD activity corresponded to α and β chains of fodrin. A band at 150 kDa was also detected by polyclonal specific antibodies to fodrin (Fig. 1C). Fodrin is easily cleaved during the preparation, giving a product of about 150 kDa (28). Fodrin was absent in fractions without inhibitory effect.

To confirm the involvement of this protein in PLD regulation, immunoprecipitation using specific fodrin antibodies was performed. As shown in Fig. 2a, fodrin was not detected by immunoblotting in the specifically immunoprecipitated fraction which has lost its ability to inhibit PLD activity (Fig. 2b).

Purification of Fodrin—Fodrin responsibility in PLD inhibition was also confirmed using purified protein extracted from bovine brain membranes according to Davis and Bennett (28). Briefly, membranes from bovine brain were prepared by homogenizing the brain in a buffer at low ionic strength at 4°C. The cytosol was then removed, and membranes were washed several times at low ionic strength (4°C). This procedure reduced the efficiency of proteases. Fodrin was then extracted from membranes at 37°C for 1 h in the same hypotonic buffer in the presence of a reducing agent, dithiothreitol. The extracted fodrin was purified under its tetrameric form with an apparent molecular mass of approximately 1,000 kDa on gel filtration. Fig. 3, A and B, shows that the peak of protein with
PLD inhibitory effect, eluted from DEAE-Sepharose, corresponds to fodrin as revealed by immunoblot analysis with specific fodrin antibodies (Fig. 3C).

**Fodrin Inhibits PLD Activity at Nanomolar Concentrations**—The inhibitory effect of pure fodrin on PLD activity was studied on HL-60 permeabilized cells either in the absence or in the presence of 20 μM GTPγS. The level of activation of PLD is mainly dependent on the number of cells present. This number determines the amount of PLD itself and of cytosolic and membrane PLD activators, such as the different small G-proteins present in the assay. To study the relative amount of fodrin necessary to inhibit PLD activity, the experiments were performed using regularly 0.5–10^6 cells/assay in the presence of different concentrations of pure fodrin. Fig. 4 reports the dose-response curve of PLD inhibition. As fodrin is purified as a tetramer (28), the concentration of the protein able to inhibit half of the GTPγS-stimulated PLD activity was estimated to be equivalent to 0.8 nM. PLD activity was fully inhibited by fodrin at concentrations higher than 1 nM. In the absence of GTPγS, PLD was only slightly active; however, the same concentrations of fodrin also inhibit, to a small extent, basal PLD activity (Fig. 4). After boiling, fodrin at 10 nM has lost all its inhibitory effect on PLD.

**Effect of Preincubation of Fodrin with PLD Substrate (PC) and/or PLD Cofactor (PIP2)—**We tested the hypothesis that fodrin may prevent the access of PLD to either its substrate (PC) or its cofactor (PIP2). As shown in Fig. 5A, preincubation of fodrin with vesicles made of PC is able to prevent PLD inhibition by fodrin only at concentrations of PC in the millimolar range, whereas PC vesicles in the absence of fodrin have no effect on choline release. Other authors have reported that the presence of PIP2 in phospholipid vesicles, is necessary for the enzyme activity *in vitro*. Fodrin β chain possesses a PH domain known to bind to PIP2. The hypothesis that fodrin inhibits PLD activity by binding PIP2 and, thus, would sequester the cofactor for PLD activation was investigated by preincubating fodrin with vesicles containing various concentrations of PC and a constant concentration of PIP2 (125 μM). As reported in Fig. 5B, such vesicles have no effect on choline release (control). Only millimolar concentrations of phospholipids inhibit fodrin effect on phospholipase D activity.

**Doses of phosphatidylcholine able to overcome fodrin effect on phospholipase D represent about 1000-fold the amount present within the cells during the reaction as estimated by measurement of phosphate present in cell aliquots and in vesicles according to Galliard *et al.* (33). Thus it seems unlikely in cellular experiments that fodrin acts on PLD by substrate or cofactor depletion.**

**Effect of Fodrin on Other Enzymes Involved in Signal Transduction: Phospholipase A2, Phospholipase C, and Adenylate Cyclase—**To check the specificity of fodrin on phospholipase D activity, we looked at the possible effect of this protein on other enzymes involved in signal transduction. Other phospholipases were chosen as they translocate toward the membrane after

![Fig. 2. Depletion of PLD inhibitory activity with fodrin antibodies.](http://www.jbc.org/)

![Fig. 3. Purification of non-erythroid fodrin and identification of its inhibitory effect on PLD activity.](http://www.jbc.org/)

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**FIG. 2.** Depletion of PLD inhibitory activity with fodrin antibodies. A cytosolic fraction containing PLD inhibitory activity, obtained after DEAE chromatography, was treated with affinity-purified fodrin antibodies (lane B) or with control rabbit Ig (lane A). The content of fodrin in both cytosolic fractions was visualized by immunoblotting in a. PLD activity was tested in the absence of cytosolic fraction (Control) and in the presence of cytosolic fractions (columns A and B) in Fig. 2b.

**FIG. 3.** Purification of non-erythroid fodrin and identification of its inhibitory effect on PLD activity. Membranes from bovine brain were prepared and fodrin was then extracted at low ionic strength. Fodrin was purified as reported (Davis and Bennett (28)) by two chromatography steps, by size exclusion on a Bio-Gel A-5m, followed by a weak anion exchange filtration (DEAE-Sepharose). Fractions eluted from the DEAE-Sepharose column were analyzed for PLD activity (A), protein (6% SDS-PAGE) (B), and fodrin (C) content as in Fig. 1.
Inhibition of Phospholipase D Activity by Fodrin

PLD Inhibition by Fodrin Is Stable—As reported in Fig. 6, fodrin at 3 nM leads to a complete inhibition of PLD activity, which can be observed already after 2.5 min (the earliest period studied) and remains stable for at least 30 min (the longest period studied). Thus, in the presence of GTPγS, which activates G-proteins, PLD can be maintained in an inactive state when fodrin is present at a sufficient concentration.

Fodrin Inhibits Different Pathways of PLD Activation—Ca^{2+} in the micromolar range stimulates, to a small extent, the basal level of PLD in permeabilized HL-60 cells. In the presence of GTPγS, this activity is highly stimulated at Ca^{2+} concentrations from 10^{-6} to 10^{-5} M. Fodrin at 1 nM has no effect on the basal level stimulated by 10^{-5} M Ca^{2+}, whereas it has a marked inhibitory effect on the GTPγS-stimulated PLD activity, which is almost totally abolished (Fig. 7A).

It has been shown that Mg-ATP is not necessary for PLD activation, but it amplifies the GTPγS response (34). We studied the effect of fodrin on the PLD activity stimulated with different concentrations of this nucleotide in the presence or in the absence of GTPγS. As reported in Fig. 7B, the highest
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**TABLE I**

| Enzymatic activity                                      | Buffer                  | Basal + GTPγS (20 μM)† | +Fodrin (3 nM)       |
|---------------------------------------------------------|-------------------------|-------------------------|----------------------|
| Phospholipase Aα (% of [3H]-labeled amino acid release of total incorporated)† | Basal + GTPγS (20 μM)† | 7.80 ± 0.83             | 2.30 ± 0.82          |
| Phospholipase C (% of phosphoinositol hydrolysis in IP3 + IP4)§ | Basal + GTPγS (20 μM)† | 12.25 ± 1.51            | 2.20 ± 1.06          |
| Adenylyl cyclase (pmol/mg/min)‡ | Basal + Mn2+ (1 mM) | 0.84 ± 0.17             | 0.64 ± 0.08          |

† As PLA2 was almost not detectable in undifferentiated HL-60 cells, fodrin effect on PLA2 activity was tested in cells previously differentiated for 2 days with dibutyryl cAMP (500 μM). Results are mean ± S.E.

‡ Results are the mean ± S.E. IP3, inositol 1,4-phosphate; IP4, inositol 1,4,5-phosphate.

§ Basal levels for PLA2 and PLC represent the enzyme activity measured in the absence of GTPγS at pCa5 with Mg-ATP (2 mM).

**TABLE II**

| Cytoskeletal protein | Buffer | Basal + GTPγS | +Fodrin (3 nM) |
|----------------------|--------|---------------|---------------|
| Control              | 100    | 96 ± 10       | 101 ± 3       |
| Fodrin (0.003 μM final) | 19 ± 7 | 101 ± 3       | 90 ± 8        |
| G actin (1.2 μM)     | 101 ± 3| 101 ± 3       | 90 ± 8        |
| F actin*             | 90 ± 8 | 90 ± 8        | 90 ± 8        |

* F actin was prepared by polymerization of G actin solution (2 mg/ml) as reported (55).

**FIG. 6.** Time course of effect of fodrin on PLD activity. GTPγS-stimulated PLD activity was measured for various periods of time in streptolysin O-permeabilized HL-60 cells in the absence (○) or in the presence of 1 nM pure fodrin (●). The PLD basal level (×) was measured in the absence of GTPγS. Results are expressed as the percentage of PtdCho hydrolysis ± S.E. and are representative of three different experiments. In this experiment, the total radioactivity in cells was 84 × 10⁶ dpm.

Effect of Fodrin Hydrolyzed in Vitro by Calpain 1 and Calmodulin on PLD Activity—It is known that Ca2+/calmodulin binds to fodrin and allows its proteolysis by calpain 1. The hydrolysis of fodrin α chain by calpain 1 occurs only in the presence of Ca2+ (35), whereas the β chain hydrolysis requires both Ca2+ and calmodulin (36). Thus, fodrin was submitted to proteolysis by incubation at 30 °C for 30 min either in the presence of EGTA, Ca2+, calpain, and calmodulin or with different combinations of these components. The inhibitory effect of the intact or proteolyzed protein was then tested on PLD activity fully stimulated by GTPγS, Mg-ATP, and Ca2+. No difference in the inhibition of PLD activity was observed between intact fodrin and this protein cut by calpain 1 with or without calmodulin (data not shown). Therefore, it is unlikely that either lysis by calpain 1 or calmodulin in the presence of Ca2+ would be a way for removing inhibition by fodrin in cells.

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**DISCUSSION**

In this study, we identify fodrin, the non-erythroid spectrin, as the first described natural inhibitor of phospholipase D. This cytoskeletal protein, which is mostly associated with plasma membrane in resting cells, is likely to play an active role in the control of a phospholipase activity involved in signal transduction.

Fodrin is a high molecular mass protein, purified as a dimer with a molecular mass of about 1000 kDa. We wondered whether the inhibitory effect detected at the earliest time of PLD measurement could be the consequence of a nonspecific steric occupancy related to the size of the protein. This hypothesis can be ruled out for the following reasons: (i) the concentration of fodrin provoking the maximal inhibition is very low, in the nanomolar range; (ii) erythroid spectrin, which shares the same molecular mass, also inhibits PLD activity but at a higher concentration (30-fold), indicating the higher specificity of fodrin in non-erythroid cells; (iii) after complete cleavage of both chains of fodrin in the presence of calpain 1, Ca2+, and calmodulin, proteolyzed proteins are still inhibitors for PLD activity (data not shown); (iv) the activity of adenylate cyclase, another enzyme involved in signal transduction and located at the plasma membrane, was not modified in the presence of fodrin in conditions which almost completely inhibit PLD activity. Thus, fodrin does not appear to inhibit PLD unspecifically by steric hindrance due to its large size. Complex interrelationships exist between the three classes of phospholipases. PA, the product of PLD activation, is stimulatory for PLA2 (37) and arachidonate products, including lysophosphatidic acid (reviewed in Ref. 38), leukotriene B4, prostaglandin F2α, and 12-hydroxyeicosatetraenoic acid (39–41) stimulate PLD. This enzyme activity requires PIP2, the PLC substrate (42), and is stimulated by diacylglycerol, the product of PLC activity (reviewed in Ref. 1). In the present work we report that the inhibitory effect of fodrin is not exclusive to PLD activity but affects also PLA2 and PLC activities. This observation might be...
explained by one or both of the two following mechanisms. 1) Fodrin might sequester phospholipids necessary for different enzyme activities. In favor of this hypothesis is the observation that all activation pathways studied are inhibited by this protein. However, our results (Fig. 5) appear to exclude the hypothesis of substrate or cofactor depletion. 2) Fodrin may act by preventing phospholipases translocation to the membrane, maintaining the enzymes under an inactive state.

Fodrin with other cytoskeletal proteins, forms a two-dimensional network at the cytoplasmic surface of plasma membranes to which it gives a structural support, a mechanical stability and determines the shape of the cells. It is also responsible for the flexibility and elasticity of the cells, necessary to achieve different physiological processes including secretion and division. Moreover, fodrin seems to play a role in specialized cell domains, in the regulation of vesicle-membrane interactions and in formation of cell-cell junctions (reviewed in Refs. 43–45). How this protein modulates PLD activity is not yet understood. Different mechanisms of action can be hypothesized.

Our study indicates that fodrin maintains PLD under an inactive state. To restore an active enzyme, post-translational modifications of the cytoskeletal protein and/or of phospholipase D itself, including proteolysis and/or phosphorylations are probably necessary.

Whether one or several domains of fodrin are involved in the regulation of phospholipase D is still unknown.

Fodrin is a rod-shaped protein composed of several domains. The complete sequences of non-erythroid α and β chains are known. Both chains of erythrocyte spectrin are mainly comprised of tandem homologous 106 residue units, flanked by nonhomologous NH2- and COOH-terminal sequences. These nonhomologous areas contain particular domains or modules such as (i) the actin binding domain at the NH2-terminal end of β chain; (ii) a PH domain (pleckstrin homologous) present only in the COOH end of non-erythroid spectrin; (iii) two EF hand domains (calcium-binding domain) at the COOH-terminal end of α chain; and (iv) a SH3 domain (Src homologous). PH and SH3 domains have been found in numerous proteins, most of them involved in signal transduction (46). In addition, it has been demonstrated recently that fodrin SH3 domain binds to amiloride-sensitive Na+ channel in epithelial cells (47), suggesting a potential effect of fodrin in maintaining proteins in specific membrane localization. It seems unlikely that the PH domain of fodrin would be the main chain sequence involved in PLD regulation, first because of the lack of modification of the fodrin inhibitory effect after incubation with PIP2 and second because of our observation that erythrocyte spectrin, which does not possess a PH domain, also exerts an inhibitory effect on PLD activity. We are currently investigating which specific domain could be responsible for PLD regulation.

Thus, fodrin appears to be a potent inhibitor of PLD activity.

### FIG. 7

**Effect of fodrin on Ca\(^{2+}\), GTP\(\gamma\)S, and Mg-ATP-stimulated PLD activity.** GTP\(\gamma\)S-stimulated PLD activity was measured at various concentrations of Ca\(^{2+}\) with Mg-ATP (2 mM) in the presence (●) or in the absence (○) of 1 nM fodrin (A); at 10\(^{-5}\) M Ca\(^{2+}\), with various concentrations of Mg-ATP in the presence (●) or in the absence (○) of 1 nM fodrin (B). PLD basal level (×) was measured in the absence of GTP\(\gamma\)S. Results are expressed as the percentage of PtdCho hydrolysis ± S.E. and are representative of three different experiments. In the experiment shown the total disintegrations/min incorporated in cells was 68 × 10\(^3\).

### FIG. 8

**Effect of fodrin on PMA- and pervanadate-stimulated PLD activity.** Before permeabilization, intact cells were incubated for 10 min at 37 °C with 1 μM PMA or 100 μM pervanadate. Control cells, PMA, and pervanadate-treated cells were then washed, and PLD activity was measured at pCa 5 in streptolysin-O-permeabilized cells in the presence of MgCl\(_2\) (2 mM) and Mg-ATP (2 mM) without addition of GTP\(\gamma\)S, in the absence (open bars) and in the presence (shaded bars) of 3 nM fodrin. Results are expressed as the percentage of choline release and are the mean ± S.D. of two separate experiments made in tritice (total disintegrations/min incorporated in untreated and treated cells were kept around 75 × 10\(^3\) (±5 × 10\(^3\)) in both experiments).
The plasma membrane via a local reorganization of the sarcoplasmic reticulum. This process is essential for the activation of PLD, which acts as an inhibitor of PLD (12). Activation of the enzyme with pure recombinant rhoA has also been shown to be involved in the control of PLD activity. Several small G-proteins involved in the control of PLD activity. Several small G-proteins have been shown to be in- volved in the regulation of PLD activity. A new concept for the understanding of PLD regulation of actin polymerization and bound to the cytoskeleton might reflect the involvement of this cellular structure

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Inhibition of Phospholipase D Activity by Fodrin: AN ACTIVE ROLE FOR THE CYTOSKELETON

Sandra Lukowski, Marie-Christine Lecomte, Jean-Paul Mira, Philippe Marin, Huguette Gautero, Françoise Russo-Marie and Blandine Geny

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Inhibition of phospholipase D activity by fodrin. An active role for the cytoskeleton.

Sandra Lukowski, Marie-Christine Lecomte, Jean-Paul Mira, Philippe Martin, Huguette Gautero, Françoise Russo-Marie, and Blandine Geny

Page 24169, Table I: This table was printed incorrectly. The correct table is shown below:

| Table I | Effect of fodrin on phospholipase A₂, phospholipase C, and adenylate cyclase activity in HL-60 cells |
|---------|-------------------------------------------------------------------------------------------------|
| Enzymatic activity                      | No addition | + Fodrin (5 nM) |
| Phospholipase A₂ (% of ³H-labeled amino acid release of total incorporated)                       | Basal level⁵ | 7.80 ± 0.83 | 2.30 ± 0.82 |
|                                                   | + GTPγS (20 µM) | 12.25 ± 1.51 | 2.20 ± 1.06 |
| Phospholipase C (% of phosphoinositol hydrolysis in IP₂ + IP₃)       | Basal level⁵ | 0.84 ± 0.17 | 0.64 ± 0.08 |
|                                                   | + GTPγS (20 µM) | 5.43 ± 0.84 | 1.35 ± 0.39 |
| Adenylate cyclase (pmol/mg/min)       | Basal level | 44.13 ± 0.26 | 47.77 ± 1.63 |
|                                                   | + Mn²⁺ (1 mM) | 149.65 ± 1.90 | 142.37 ± 11.93 |

⁵ As PLA₂ was almost not detectable in undifferentiated HL-60 cells, fodrin effect on PLA₂ activity was tested in cells previously differentiated for 2 days with dibutyryl cAMP (500 µM). Results are the mean ± S.E.
⁶ Basal levels for PLA₂ and PLC represent the enzyme activity measured in the absence of GTPγS at pCa5 with Mg-ATP (2 mM).
⁷ Results are mean ± S.E. IP₃, inositol 1,4-phosphate; IP₃, inositol 1,4,5-phosphate.

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Evidence for a phorbol ester-insensitive phosphorylation step in capacitative calcium entry in rat thymic lymphocytes.

Ian Marriott and Michael J. Mason

Pages 26734, 26735, and 26736, legends to Figs. 2, 3, and 4: p values quoted should read p ≤ 0.05 and not p ≥ 0.05 as stated in the legend.

Pages 26735 and 26736, legends to Figs. 3 and 4: Sentence beginning “C, unidirectional uptake rates derived from data presented in A” should read: “C, unidirectional uptake rates derived from data presented in B.”

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Anti-Ig-induced calcium influx in rat B lymphocytes mediated by cGMP through a dihydropyridine-sensitive channel.

Amir A. Sadighi Akha, Nicholas J. Willmott, Kieran Brickley, Annette C. Dolphin, Antony Galione, and Simon V. Hunt

The first author's name was listed incorrectly in the author index. The author's surname is Sadighi Akha and should have been cited as Sadighi Akha, Amir A.

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