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Selective Estrogen Receptor (ER) Modulators Differentially Regulate Phospholipase D Catalytic Activity in ER-Negative Breast Cancer Cells

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ABSTRACT

Recent successes in the pharmacotherapeutic treatment of breast cancer are associated with the use of selective estrogen receptor modulators. Two commonly prescribed pharmaceuticals in this class, tamoxifen and raloxifene, have been shown to have effects through estrogen receptor (ER)-independent mechanisms. Hyperactivation of phospholipase D (PLD) in certain tumor-derived cell lines have been reported, and recent findings suggest a role for PLD in transformation and metastasis. In the present study, we compare the effects of tamoxifen and raloxifene on PLD in the ER-positive mammary epithelial cell line MCF-12A, and the ER-negative, highly tumorigenic mammary carcinoma cell line MDA-MB-231. Our data demonstrate that tamoxifen and raloxifene have differential effects on PLD catalytic activity. Tamoxifen stimulates PLD in both ER-positive and -negative cells in vivo, whereas raloxifene inhibits PLD activity in these same cell types. In addition, we show that the active metabolite 4-OH-tamoxifen can be used to pharmacologically discriminate the two isoforms of PLD, through a stimulatory effect on PLD1 and an inhibitory effect on PLD2. Using recombinant PLD1, we show stimulation by tamoxifen requires a factor present in Sf21 insect cells that is not required for inhibition of PLD1 by raloxifene. Furthermore, tamoxifen stimulation and raloxifene inhibition of PLD activities are independent of the amino-terminal portion of PLD1 (amino acids 1–324). Knowledge of the mechanisms of action of these drugs on PLD may provide insights into the pharmacological action of these drugs and the role of PLD in some cancers.

The use of selective estrogen receptor modulators (SERMs) has proven to be a successful strategy in the treatment of breast cancer. Two of the most commonly prescribed SERMs are tamoxifen and raloxifene (Evista). Studies have shown the ability of both tamoxifen (Fisher et al., 1998) and raloxifene (Cummings et al., 1999) to decrease the incidence of breast cancer in high-risk patients. The use of these compounds arises from their ability to compete for binding at the estrogen receptor (ER) (Skidmore et al., 1972; Jordan and Koerner, 1975), and elicit a distinct conformation of the receptor different from endogenous estrogen (Brzozowski et al., 1997; Paige et al., 1999). Although the binding of tamoxifen and raloxifene are indistinguishable at the level of the receptor, there is pharmacological evidence to suggest a difference in the effects of these two compounds. Patients undergoing tamoxifen treatment exhibit an increased incidence of endometrial cancer (Fisher et al., 1998) that is not present in patients on a raloxifene regimen (Cummings et al., 1999). This suggests that not only could tamoxifen and raloxifene work through distinct mechanisms, or have differential effects on the same target, but that these effects may be independent of ER binding.

SERMs have been shown to have antiproliferative effects in some patients with mammary tumors that are ER-negative (Plowman 1993). Several intracellular binding proteins have been identified for estradiol (Anderson et al., 1986), and it seems likely that ancillary proteins modulate the intracellular actions of SERMs as well. There is evidence to support a direct interaction of tamoxifen with the antiestrogen binding site protein AEBS (Chouvet and Saez, 1984), the P-glycoprotein efflux pump (Leonessa et al., 1994), calmodulin (Lopes et al., 1990), and the regulatory domain of protein kinase C (PKC) (Bignon et al., 1991). Interestingly, this PKC interaction is complex and groups have observed either increased (Bignon et al., 1991) or decreased (O'Brian et al., 1986) PKC activity depending on precise conditions.

Various studies suggest that phospholipase D (PLD) activ-

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ABBREVIATIONS: SERM, selective estrogen receptor modulator; ER, estrogen receptor; PKC, protein kinase C; PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; PEtOH, phosphatidylethanol; Sf21, *Spodoptera frugiperda* 21; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TTBS, Tris-buffered saline/Tween 20; Arf, ADP-ribosylation factor.

ity may play a mechanistic role in cellular transformation. Elevated levels of PLD have been demonstrated in human breast cancer tissues (Noh et al., 2000), human gastric carcinoma cells (Uchida et al., 1999), and tumor cells from rats with dimethylhydrazine dihydrochloride-induced colon cancer (Yoshida et al., 1998). Furthermore, elevated PLD activity, specifically by the isoform PLD2, was reported in human colon adenocarcinoma cells, human breast adenocarcinoma cells (Fiucci et al., 2000) and human renal cancers (Zhao et al., 2000). Stable cell lines overexpressing PLD1 and PLD2 in fibroblasts exhibit anchorage-independent growth, up-regulation of matrix metalloprotease-9 activity, and induce tumorigenesis when transplanted into nude mice (Min et al., 2001).

PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline (reviewed in Singer et al., 1997; Exton, 1999; Frohman and Morris, 1999). Increased levels of PA in the cell have been implicated in signaling cascades that regulate cell growth and metastasis. One likely target of PA signaling includes mitogenic activation of mTOR, the mammalian target of rapamycin, which regulates cell growth and proliferation through the initiation of mRNA translation and is activated by an accumulation of PA through a PLD-dependent pathway (Fang et al., 2001). Hydrolysis of PA by phospholipase A2 generates the potent mitogen lysophosphatidic acid, and in a screen of patients with ovarian cancers, increased serum levels of lysophosphatidic acid correlated with the degree of cancer malignancy (Xu et al., 1995; Westermann et al., 1998).

Interestingly, it has been shown that tamoxifen can stimulate cellular PLD activity through an ER-independent mechanism (Kiss, 1994). The proposed mechanism of this tamoxifen stimulation was through the PLD activator PKC, and we wanted to further explore this interaction in vitro. The tamoxifen effects on PLD are also compared with that of raloxifene both in the ER-positive, normal mammary epithelial cell line MCF-12A and the ER-negative, highly transformed mammary carcinoma cell line MDA-MB-231. Data presented here demonstrate that tamoxifen and raloxifene have differential effects on PLD independent of the ER in vivo, and in vitro studies support and extend this finding, suggesting that this regulation is through two different mechanisms. In addition, we provide evidence demonstrating a pharmacological distinction between PLD1 and PLD2 using the active tamoxifen metabolite 4-OH-tamoxifen. This finding provides us with a pharmacological tool to help identify the active isoform of PLD in carcinoma cells in vivo.

Considering the evidence supporting the ER-independent mechanisms of action of SERMs, and specifically the intracellular binding capabilities of tamoxifen, we hypothesize that the distinct effects of tamoxifen and raloxifene, are through the differential regulation of PLD isoenzymes.

Materials and Methods

Materials

The SERMs tamoxifen free acid, tamoxifen citrate salt, and 4-hydroxytamoxifen were obtained from Sigma-Aldrich (St. Louis, MO), and raloxifene (Evista) was obtained from Eli Lilly and Company. Stocks of 10 mM tamoxifen and 10 mM 4-OH-tamoxifen were made in 95% ethanol, whereas stocks of 10 mM tamoxifen citrate and 15 mM raloxifene were made in 70% ethanol. All SERM working stocks were stored at −20°C. Phorbol 12-myristate 13-Acetate (PMA) was obtained from Sigma. Calcium ionophore A23187, cholera toxin, *Streptomyces chromofuscus* PLD, *Bacillus cereus* PLC, and hydrocortisone were obtained from Calbiochem (San Diego, CA). Most tissue culture supplies [DMEM, Ham's F-12 medium, human recombinant EGF, insulin (bovine, zinc), penicillin, streptomycin, amphotericin B, and horse serum] were obtained from Invitrogen (Carlsbad,CA). Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA). [^{3}H]Myristic acid was obtained from PerkinElmer Life Sciences (Boston, MA). UTP was obtained from Roche Applied Science (Indianapolis, IN).

Cell Culture

The ER-positive, nontumorigenic, human mammary epithelial cell line MCF-12A was obtained from the American Type Culture Collection (Manassas, VA). These cells, widely characterized as a model for normal mammary epithelial cells, were continuously cultured in 1:1 DMEM/Ham's F-12 medium, supplemented with 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.01 ng/ml insulin, 500 ng/ml hydrocortisone, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5% horse serum. The ER-negative, highly transformed, tumorigenic human mammary epithelial cell line MDA-MB-231 was also obtained from American Type Culture Collection. These cells were maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% fetal bovine serum. Both cell lines were maintained in a controlled, humidified environment of 37°C and 5% CO_2.

Measurement of Transphosphatidylation in MCF-12A and MDA-MB-231 Cells

The in vivo PLD activities were measured essentially as described elsewhere (Balboa et al., 1994; Cohen and Brown, 2001). Briefly, cells were plated in six-well culture plates (#3516, Costar, Corning Glassworks, Corning, NY) at a density of 1×10^6 (MCF-12A) or 0.8×10^6 (MDA-MB-231) cells per well, in serum-containing medium. The difference in seeding density was to allow the same number of cells to be used despite the faster growth rate of the MDA-MB-231 cells. Twenty-four hours after seeding and at ~85% confluence, the cells were serum starved (MCF-12A cells were also deprived of EGF) for a total of 72 h. For the last 20 h of serum starvation, the cells were labeled with [^{3}H]myristic acid (10 μCi/ml). Subsequently, all cells were washed in fresh media (serum-free/EGF-free) and assayed for 30 min at 37°C in the presence of 2% ethanol, to measure the transphosphatidylation reaction mediated by PLD. In the cases of SERM addition, the final ethanol concentration was 2.1%. Assay conditions were terminated by placing the cell plates directly on ice, removing the assay media, and bathing the cells in 600 μl of ice-cold 0.1 N HCl/MeOH (1:1). Cells were then scraped into 300 μl of ice-cold chloroform for lipid extraction. Concentrated lipid samples were loaded onto a Linear-K6 thin-layer chromatography plate (60-Å pore size, Whatman Inc., Clifton, NJ) and phosphatidylethanol (PEtOH) was resolved using a resolving system containing a 10:2:4:2:1 chloroform/methanol/acetone/acetic acid/water mixture. After at least 24 h of solvent evaporation, the thin-layer chromatography plate was exposed to a phosphor screen to enhance the tritium-labeled transphosphatidylation products of activated PLD.

Cell Growth Assay

Mammalian cell lines MCF-12A and MDA-MB-231 were seeded in 12-well tissue culture plates (35-3043; Falcon Plastics, Oxnard, CA) at 2×10^4 cells/well in 1 ml of complete growth media. The following day (day 0), the media was replaced with growth media supplemented with 1% serum and either vehicle (0.5% EtOH) or 4-OH-tamoxifen (1 μM). Media and treatment were replenished every 24 h. Starting on day 2, cells were released with trypsin, stained with Trypan blue, and counted using a hemacytometer.

Recombinant Protein Expression

A new baculovirus construct of full-length PLD1 was generated for these studies. Full-length PLD1 was cloned from pCMV3 PLD1 and inserted into pCR Blunt. Using an engineered 5′ restriction site (*Sal*I) and a 3′ site from the vector (*Not*I), PLD1 was subcloned into the pFastBac baculovirus vector (Invitrogen). Baculovirus production was carried out according to the Bac-to-Bac protocol (Invitrogen). A partially purified PLD1 was generated essentially as described in Walker et al., 2000. Monolayers of *Spodoptera frugiperda* 21 (Sf21) cells were infected with baculovirus encoding human PLD1. After a 72-h infection, the cells were harvested, washed twice in 2 ml solution F (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 mM EDTA), and lysed by nitrogen cavitation at 4°C for 60 min at 1100 p.s.i. Membranes and cytosol were separated by centrifugation at 174,000*g* for 1 h. The cytosol was isolated and loaded onto a 5-ml High Trap SP-Sepharose column (Amersham Bioscience, Piscataway, NJ) equilibrated with Solution F. The cytosol load was eluted with a 36-ml gradient from 100 mM to 1.0 M NaCl in solution F and fractions were assayed for PLD activity.

An amino-terminal truncation of PLD1 (amino acids 325-1074) was initially cloned from pCMV3 PLD1 and inserted into pCR Blunt (Invitrogen). Using engineered restriction sites (*Bgl*II-*Xba*I) from the initial polymerase chain reaction step, PLD1 (325–1074) was subcloned from pCR Blunt into the baculovirus transfer vector, pVL1392 (BD Biosciences PharMingen, San Diego, CA). PLD1 (325–1074) baculovirus was generated after transfection of Sf21 cells using the BaculoGold transfection kit (BD Biosciences PharMingen). Protein was expressed and purified as described previously (Walker et al., 2000). The original cDNA encoding PLD1 was kindly supplied by Michael Frohman and Andrew Morris at SUNY Stony Brook, NY.

Full-length human PLD2 expressing baculovirus was expressed in Sf21 cells, and the membrane-bound fraction of protein was isolated as described above. The cDNA encoding PLD2 was a generous gift from David Lambeth at Emory University. PKC-α, RhoA, and all Cdc42 recombinant proteins were expressed in Sf21 cells and chromatographically purified as described previously (Walker et al., 2000). Recombinant Arf-1 was coexpressed with *N*-myristoyltransferase in *Escherichia coli*, partially purified by anion exchange on a DEAE-Sepharose column, and subsequently on an AcA44 gel filtration column essentially as described elsewhere (Randazzo et al., 1992; Brown and Sternweis, 1995).

Exogenous Substrate Assays

Exogenous lipid vesicles were prepared as described previously (Brown et al., 1995; Walker et al., 2000). SERM ethanol stocks were warmed to 37°C before use. The order of addition of assay components was critical, because early experiments (data not shown) suggested the PLD enzyme could become unstable in the presence of a high ethanol concentration: PLD source, activators (as indicated), guanine nucleotide, reaction buffer, drug, and liposomes. The final concentration of ethanol in all vehicle or treatment conditions was 1.2%. All assays were conducted at 37°C for 30 min with 10 μM guanosine 5′-*O*-(3-thio)triphosphate. PLD activity was measured essentially as described elsewhere (Brown et al., 1993).

Chromatographic Isolation of the Tamoxifen Stimulating Factor

All purification procedures were performed at 4°C. Uninfected monolayers of Sf21 cells were grown to confluence in 10× T-150 tissue culture flasks (430823; Corning). Cells were harvested, washed twice in 2 ml of solution F, and lysed by nitrogen cavitation at 4°C for 60 min at 1100 p.s.i. Membranes and cytosol were separated by spinning at 174,000*g* for 1 h. The cytosol was isolated and loaded onto a 5-ml High Trap SP-Sepharose column (Amersham Biosciences) equilibrated with Solution F. The cytosol load was eluted with a 36-ml gradient from 100 mM to 1.0 M NaCl in solution F. Fractions were collected (1 ml). Each fraction was added back to 0.2 μg SP-Sepharose–purified PLD1 in the presence of 20 μM tamoxifen citrate. Restoration of tamoxifen activity was isolated to fractions 2–6. These fractions were pooled and concentrated to 900 μl using a PM10 membrane. Next, 850 μl of the concentrated factor was loaded onto a 70-ml bed volume AcA44 gel filtration column equilibrated with 100 mM NaCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0. A total of 105 ml (70 fractions of 1.5 ml each) was collected at a flow rate of 0.2 ml/min. All other fractions were added back to 0.2 μg SP-Sepharose purified PLD1 in the presence of 20 μM tamoxifen citrate. The peak of restored tamoxifen activity was isolated to fractions 32 to 36, and pooled.

Western Blot Analysis

PLD Expression in Whole Cell Lysates. Mammalian cell lines MCF-12A and MDA-MB-231 were grown to 100% confluence in three 150-mm cell culture dishes (430599; Corning) and lysed by hypotonic lysis. Confluent monolayers were washed in ice-cold PBS, scraped into ice-cold PBS with a razor blade, and pelleted. After a series of washes in wash buffer (145 mM NaCl, 1 mM EGTA, 5 mM HEPES), the cells equilibrated for 45 min in lysis buffer (5 mM Tris-HCl, 1 mM EGTA, 1 mg/ml Pepstatin, 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 134 mM phenylmethylsulfonyl fluoride) at 4°C. Using a chilled Kontes tissue grinder (Fisher Scientific, Pittsburgh, PA), the cells were lysed in 0.5 ml of lysis buffer by plunging 20 times, and the resulting lysate was cleared of nuclei and unbroken cells. Equivalent protein concentrations (135 μg) were subjected to 8% SDS-PAGE. The resolved protein bands were transferred onto a PVDF membrane and blocked with 5% nonfat milk/TTBS overnight. The membrane was subsequently probed with either a monoclonal anti-PLD1 antibody for 45 min or a monoclonal anti-PLD2 antibody overnight at 4°C. After a series of washes in TTBS, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min. After another series of washes in TTBS, the membrane was developed with ECL (Amersham Biosciences) and exposed to X-ray film (Eastman Kodak, Rochester, NY) to visualize the immunoreactive bands. The monoclonal PLD2 antibody was kindly supplied by Yasunori Kanaho at the Tokyo Metropolitan Institute of Medical Science.

Cytosolic Factor. Samples were taken from fractions 31 to 38, and equivalent volumes were subjected to 12% SDS-PAGE. The resolved protein bands were transferred onto a PVDF membrane and blocked with 5% nonfat milk/TTBS overnight. The membrane was probed with a monoclonal anti-Arf antibody, and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody. The membrane was then washed and developed as described above. The monoclonal Arf antibody was kindly supplied by Richard Kahn (Emory University, Atlanta, GA).

[^{3}H]GDP Exchange Assay

The GDP dissociation assay was carried out by the filter binding method at 22°C essentially as described previously (Hart et al., 1991), with final concentrations of 1 μM Cdc42 wild-type, 100 μM nonradioactive GDP, 20 μM tamoxifen citrate or raloxifene, vehicle (1.2% EtOH), or 20 mM EDTA, as indicated.

Data Analysis

Statistical analysis of the data from the 7-day growth assays and 30-min exogenous substrate assays were done using two sample *t* tests. The conventional 0.05 probability level for rejection of the null hypothesis was used in all tests of significance.

Results

Constitutive PLD Activity in the Mammary Carcinoma Cell Line MDA-MB-231. Comparison of the nontumorigenic, ER-positive mammary epithelial cell line MCF-12A and the highly transformed, tumorigenic, ER-negative

mammary carcinoma cell line MDA-MB-231 show the carcinoma cells have a constitutive PLD activity that is not found in the normal, nontumorigenic cells (Fig. 1A). Although the enzyme PLD is present in both cell lines and activity can be measured by way of the transphosphatidylation assay upon stimulation with 1 μM PMA, the mammary carcinoma cells themselves possess a constitutive PLD activity. To delineate the prevalent isoform(s) of PLD present in these cells, a Western blot analysis of whole cell lysates of both MCF-12A and MDA-MB-231 cells was done for PLD1 (Fig. 1B) and PLD2 (Fig. 1C). Although both cell lines show expression of PLD1, only the MDA-MB-231 cells express a detectable level of PLD2.

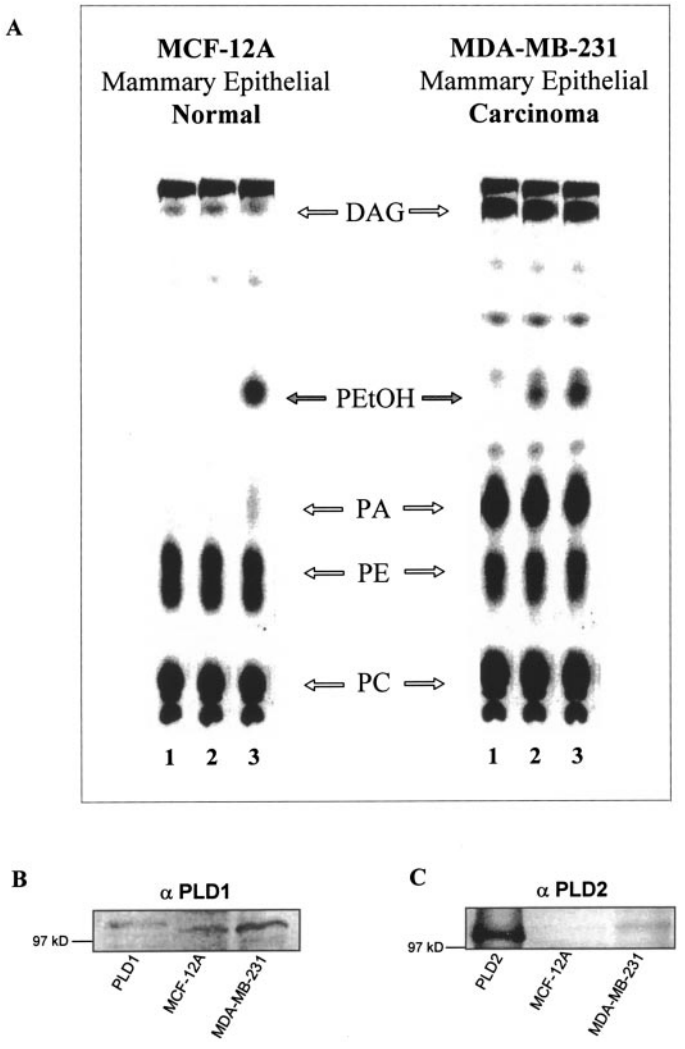

Fig. 1. A, Comparison of the lipid profiles resolved by thin-layer chromatography of the estrogen receptor positive, normal human mammary epithelial cell line MCF-12A and estrogen receptor negative, highly transformed human mammary epithelial cell line MDA-MB-231. PLD activity is measured through a transphosphatidylation reaction in intact cells at 95% confluence in a 30-min assay. Lane 1 shows PLD activity in the absence of 2% ethanol. Lane 2 shows activity in the presence of 2% ethanol. Lane 3 shows activity in the presence of 2% ethanol and maximally stimulated with 1 μM PMA. Note the high levels of PLD activity (PEtOH) and increased levels of PA in the MDA-MB-231 cells compared with the MCF-12A normal mammary cells. B and C, Western blot analysis of whole-cell lysates (135 μg) from MCF-12A and MDA-MB-231 cells for PLD1 (B) or PLD2 (C). Lysates were subjected to 8% SDS-PAGE, transferred to a PVDF membrane and processed as described in *Materials and Methods*. The PLD1 standard is protein overexpressed in Sf21 cells and chromatographically enriched over three columns, SP-Sepharose, AcA44, and Heparin. The PLD2 standard is the membrane fraction of protein overexpressed in Sf21 cells. Both MCF-12A cells and MDA-MB-231 cells express PLD1, however the level of expression is higher in MDA-MB-231 cells. Note that only MDA-MB-231 cells express a detectable level of PLD2. DAG, diacylglycerol; PE, phosphatidylethanolamine.

SERMs Differentially Regulate Endogenous PLD Activity in Mammary Epithelial Cells. Endogenous treatment of cells with 20 μM tamoxifen citrate has a stimulatory effect on the PLD activity measured as PEtOH production, in both the ER-positive MCF-12A and ER-negative MDA-MB-231 cells (Fig. 2A). The same effects were observed with 20 μM tamoxifen free acid (data not shown). Endogenous treatment of both cell types with raloxifene (20 μM) causes an inhibition of PLD activity; most notably, it inhibits the constitutive activity in the highly transformed, ER-negative MDA-MB-231 cells. In addition to inhibiting the constitutively active PLD in MDA-MB-231 cells, raloxifene also inhibits stimulated PLD activity in both cell types. Stimulation of PLD through three distinct mechanisms using PMA, the calcium ionophore A23187, and UTP is markedly decreased in both the normal, ER-positive MCF-12A cells (Fig. 2B), and the highly transformed, ER-negative MDA-MB-231 cells (Fig. 2C). This inhibition is not seen with tamoxifen (data not shown).

Interestingly, treatment with the active metabolite 4-OH-tamoxifen elicits differential effects on PLD activity in these two cell types (Fig. 2A). Addition of 4-OH-tamoxifen (20 μM) to the MCF-12A cells results in a stimulation of PLD activity similar to tamoxifen citrate but decreases the PLD activity of MDA-MB-231 cells. Chronic exposure to 4-OH-tamoxifen (1 μM) also causes phenotypic differences in MCF-12A (Fig. 2D) and MDA-MB-231 cells (Fig. 2E). In a 7-day growth assay, 4-OH-tamoxifen has significantly different effects on cell growth in MCF-12A cells compared with MDA-MB-231 cells. When treated with vehicle, MCF-12A cells reach 100% confluence on day 6 and cease to grow; however, treatment with 4-OH-tamoxifen allows proliferation to continue, causing the cell count on day 7 to be significantly higher than with vehicle. In contrast, chronic treatment of MDA-MB-231 cells with 4-OH-tamoxifen causes a significant decrease in cell proliferation starting on day 4 and continuing through day 7 compared with vehicle. These findings are consistent with PLD activity having a role in cell growth, and support the differential regulation of PLD by 4-OH-tamoxifen in these two cell types.

SERMs Differentially Regulate Recombinant PLD1 and PLD2. To further characterize the mechanisms of action of these SERMs on PLD, we examined the isoform specificity using recombinant PLD1 and PLD2. These four compounds were titrated over membrane preparations isolated from Sf21 cells overexpressing either PLD1 (Fig. 3A) or PLD2 (Fig. 3B). In an in vitro assay measuring the hydrolytic activity of PLD on liposomes, we found that both tamoxifen free acid and tamoxifen citrate can stimulate the activity of both PLD1 and PLD2. This in vitro stimulation can be seen at 10 μM, a concentration previously reported in breast tumors of some patients undergoing tamoxifen therapy (Daniel et al., 1981). Because both preparations of tamoxifen (free acid and citrate) gave similar results, all subsequent experiments were conducted using tamoxifen citrate. In contrast, raloxifene had an inhibitory effect on both PLD1 and PLD2. This inhibition can be reproducibly observed at 10 μM, exhibiting a

dose-dependent decrease with an IC_{50} of approximately 4 μM. Interestingly, the active metabolite of tamoxifen, 4-OH-tamoxifen, has differential effects on PLD1 and PLD2. This compound can stimulate the hydrolytic activity of PLD1, and inhibit the activity of PLD2. This is the only compound described to date that can distinguish the mammalian PLD isoforms. These results are reminiscent of the in vivo effects of 4-OH-tamoxifen on MCF-12A cells and MDA-MB-231 cells in Fig. 2A. Taken together, this suggests the dominant activity in MDA-MB-231 mammary carcinoma cells is PLD2. In subsequent analysis, the in vitro PLD activity was measured from MDA-MB-231 cell lysates in the presence of 4-OH-tamoxifen, tamoxifen citrate, and raloxifene. Tamoxifen citrate stimulated PLD activity in MDA-MB-231 lysates, but 4-OH-tamoxifen and raloxifene decreased PLD activity (data not shown). These findings, along with the in vivo experiments and western blot analysis (Fig. 1C, 2A and 2E) suggest that PLD2 is the hyperactive isoform of PLD in the MDA-MB-231 mammary carcinoma cells, and further indicates the ability of 4-OH-tamoxifen to differentiate between PLD isoforms. To test these compounds for any nonspecific effects on the integrity of the liposomes used in the in vitro assay, these same titrations were performed using a bacterial PLD from

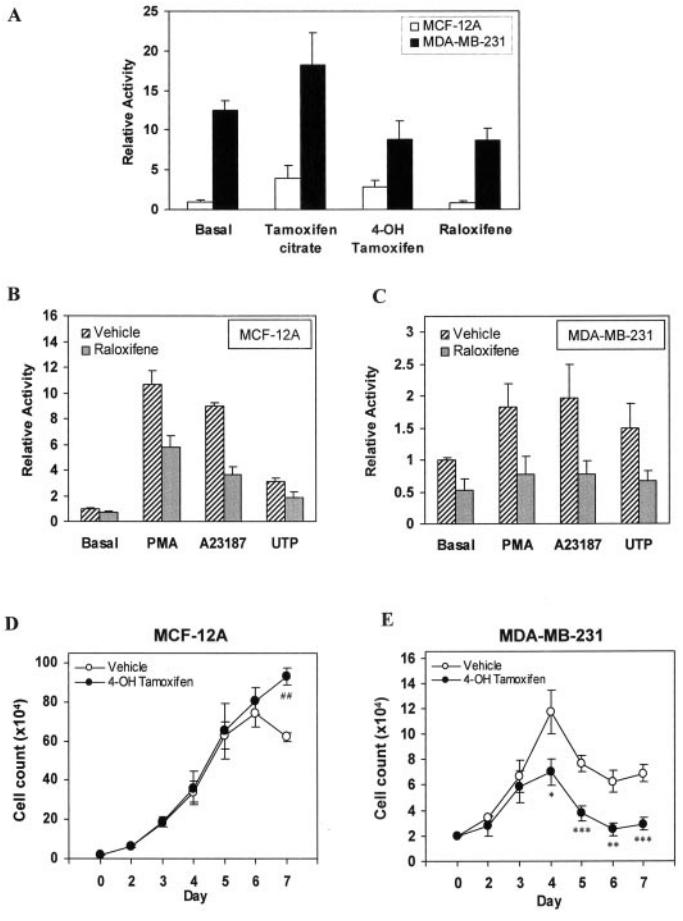

Fig. 2. A, graph represents mean ± S.E. (expressed as relative activity in arbitrary units) from three independent 30-min assays on intact cells at 95% confluence in the presence of 2% ethanol. The basal PLD activity of the ER-positive MCF-12A normal mammary epithelial cells (□) was normalized to a value defined as 1. The ER-negative MDA-MB-231 cells (■) have constitutive levels of PLD activity 12.5-fold higher than that of the ER-positive MCF-12A cells. Tamoxifen citrate (20 μM) stimulates PLD in both the ER-positive MCF-12A cells and the ER-negative MDA-MB-231 cells, and raloxifene (20 μM) inhibits the basal activities of both cell types. Addition of 4-OH-tamoxifen (20 μM) differentially affects the PLD activity in MCF-12A cells and MDA-MB-231 cells. PLD activity is stimulated in MCF-12A cells by 4-OH-tamoxifen, but is inhibited in MDA-MB-231 cells. B and C, graphs represent mean ± S.E. (expressed as relative activity in arbitrary units) from three independent 30-min assays on intact cells at 95% confluence in the presence of 2% ethanol. The basal PLD activity of the ER-positive MCF-12A normal mammary epithelial cells (B) and the ER-negative MDA-MB-231 mammary carcinoma cells (C) were normalized to a value defined as 1. Addition of 20 μM raloxifene (▨) inhibits the endogenous basal PLD activity as well as PLD activity stimulated by the addition of PMA (300 nM), A23187 (1 μM), or UTP (100 μM) in both cell types. Cells in B and C were pretreated with raloxifene 10 min before 30-min agonist additions. D and E, Growth assay of MCF-12A (D) and MDA-MB-231 cells (E) in the presence of 4-OH-tamoxifen (1 μM). Graphs represent the number of cells (mean ± S.E.) from two assays done in triplicate. Cells started at 2×10^4 per well in a 12-well plate in growth media containing 1% serum and were replenished with fresh media and treatment every 24 h. Chronic exposure to 4-OH-tamoxifen (1 μM) has a significant effect on sustaining the growth of MCF-12A past 100% confluence at day 6. However, 4-OH-tamoxifen has the opposite effect in MDA-MB-231 cells, inhibiting growth as early as day 4. Significantly greater than vehicle, ##, $P < 0.01$. Significantly less than vehicle *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

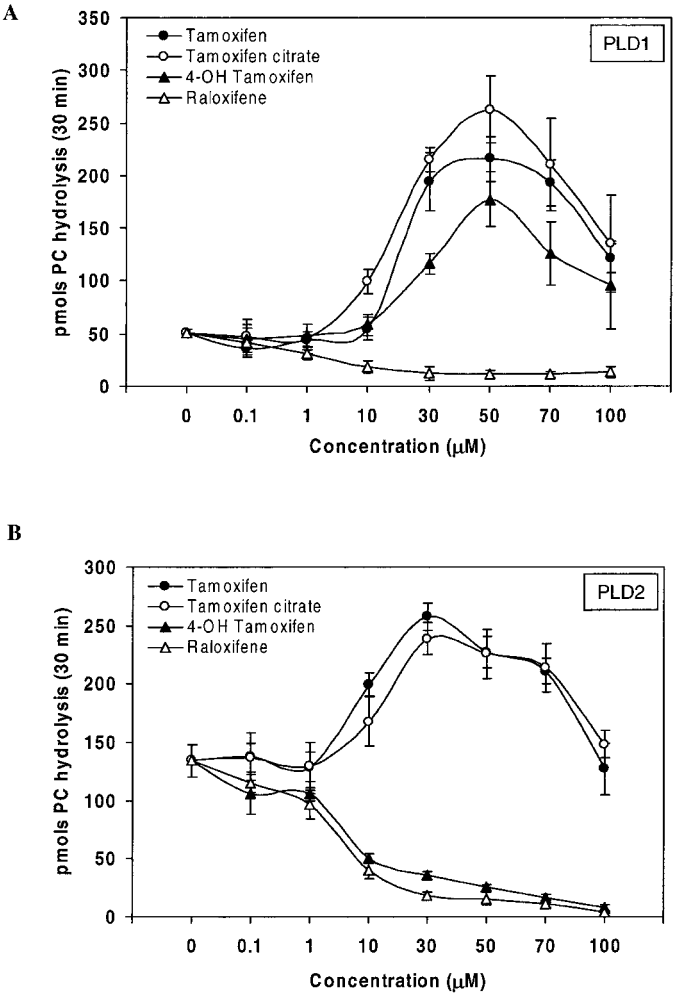

Fig. 3. Titration curves of SERMs on 5 μg of membrane protein from Sf21 cells overexpressing PLD1 (A) or PLD2 (B). PLD activity is measured in the absence of exogenously added activators through PC hydrolysis on liposomes in vitro, as described in *Materials and Methods*. Data are mean ± S.E. from dose-response curves generated in three independent assays. Tamoxifen (●) and tamoxifen citrate (○) stimulate activation of both isoforms of PLD, while raloxifene (△) inhibits both PLD1 and PLD2. 4-OH-tamoxifen (▲) stimulates PLD1 activity but inhibits PLD2, demonstrating an ability to discriminate between these PLD isoforms.

S. chromofuscus, and a PC-specific bacterial PLC from *B. cereus*. These bacterial lipases hydrolyze PC independent of the regulation by mammalian activators, and as such control for nonspecific effects of the SERMs on liposome structure or substrate availability that could interfere with lipase catalyzed PC hydrolysis. Neither tamoxifen, 4-OH-tamoxifen, nor raloxifene had any significant effect on the catalytic activity of these nonmammalian phospholipases (data not shown).

Reconstitution of a Cytosolic Factor Required for PLD Stimulation by Tamoxifen. To show specificity of the SERMs for PLD in the absence of endogenous activators present in membrane preparations, we measured the in vitro hydrolytic activity of a partially purified preparation of PLD1. Tamoxifen citrate (20 μM) was added to either Sf21 cytosol overexpressing PLD1, or to a partially purified preparation of PLD1 (Fig. 4A). Both PLD1 preparations contain a catalytically active enzyme stimulated by 1 μM Arf-1 compared with basal activity. Whereas the cytosolic fraction of PLD1 can be stimulated through the addition of tamoxifen citrate, the partially purified peak of PLD1 cannot. This suggests the chromatographic removal of a factor that is necessary for tamoxifen to stimulate PLD1. This factor was found to be common and not limited to mammary epithelial cells, because it is also present in uninfected Sf21 cells. Restoration of the tamoxifen stimulation of peak PLD1 was achieved through chromatographic isolation of the factor on an SP-Sepharose cation exchange column. The tamoxifen-stimulating factor was further resolved on an AcA44 gel filtration column and identified by reconstitution of the factor with partially pure PLD1 and tamoxifen. Chromatographic resolution of the factor revealed a peak of restored stimulation by 20 μM tamoxifen citrate between fractions 32 to 38 (Fig. 4B). Proteins contained in these fractions primarily ranged in size from 14 to 29 kDa by silver stain. Western blot analysis of this peak revealed the presence of Arf, when probed with an anti-Arf antibody (Fig. 4B, above). Addition of this factor alone to peak PLD1 did not significantly stimulate PLD activity (data not shown), suggesting that the concentration of endogenous Arf was not sufficient to activate PLD, but was sufficient to augment tamoxifen stimulation. To confirm this finding, purified recombinant Arf-1 was added to peak PLD1 in the absence or presence of 20 μM tamoxifen citrate (Fig. 4C). Note that tamoxifen cannot stimulate peak PLD1 alone, but greatly enhances PLD stimulation in the presence of recombinant Arf-1, consistent with the tamoxifen-stimulating factor in Sf21 cytosol. It is interesting to the note the effects of tamoxifen on PLD activity are analogous to the protein-factors (e.g., Cdc42) found to modulate PLD (Singer et al., 1995). The addition of tamoxifen alone has minimal or modest effects on PLD catalytic activity, but in combination with Arf the stimulation is pronounced. This synergistic effect of tamoxifen and Arf is significantly higher than with Arf alone, producing a fold stimulation of PLD activity of 26.7 with Arf and tamoxifen, versus 8.9 with Arf, or 1.6 with tamoxifen (values calculated from Fig. 4C).

Mechanism of Stimulation of PLD by Tamoxifen. Because tamoxifen in the presence of the monomeric G-protein Arf stimulates partially purified PLD1, we explored whether this mechanism was conserved with other monomeric G-proteins and other activators of PLD, and compared this to raloxifene (Fig. 5A). Using an in vitro measurement of PLD1 activity on liposomes, we compared the effects of tamoxifen citrate (20 μM) and raloxifene (20 μM) on peak PLD1 basal activity, and in the presence of purified recombinant RhoA (1

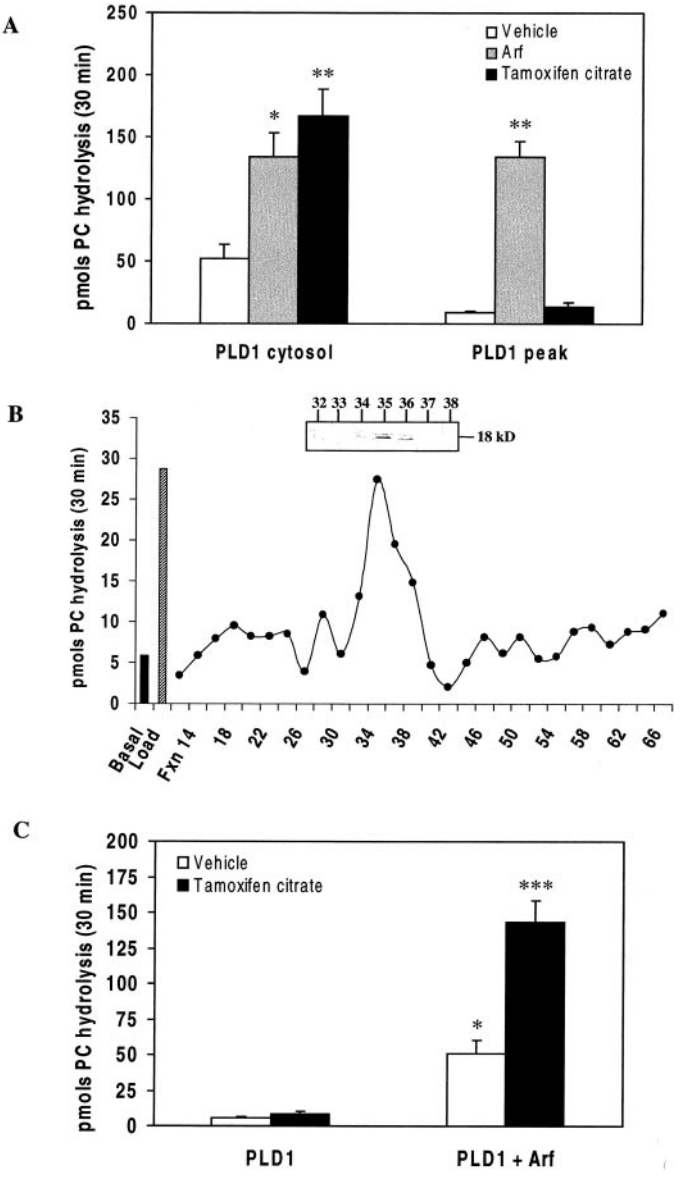

Fig. 4. Data for were collected using a 30-min in vitro assay measuring activity of recombinant PLD1 on liposomes. A, graph represents mean ± S.E. of six independent assays on 8 μg of cytosolic protein from Sf21 cells overexpressing PLD1, or 0.2 μg of PLD1 enriched by cation exchange chromatography. Both PLD1 preparations contain a catalytically active enzyme stimulated by 1 μM Arf (▨) compared with vehicle (□), however stimulation by 20 μM tamoxifen (■) requires a factor present in Sf21 cells that is removed during protein purification. Significantly greater than vehicle, *, $P < 0.05$; **, $P < 0.01$. B, graph illustrates the resolution of a concentrated cytosolic factor pool over an AcA44 gel filtration column. Individual fractions were isolated and added back to 0.2 μg of partially purified PLD1 in the presence of 20 μM tamoxifen citrate (●). The tamoxifen stimulation was restored with the factor presented in the load (▨) compared with the basal stimulation (■). Chromatographic resolution of the factor reveals a peak of stimulation between fractions 32 and 38. Proteins contained in these fractions ranged in size from 14 to 29 kDa by silver stain on SDS-PAGE. As shown, Western blot reveals concurrent Arf-immunoreactivity in this fraction pool. C, data are mean ± S.E. from 4 experiments done in duplicate. Addition of purified recombinant Arf-1 (1 μM) to 0.2 μg of a partially purified preparation of PLD1, restores and enhances stimulation by tamoxifen citrate (20 μM) (■) compared with vehicle (□). Significantly greater than vehicle without Arf, *, $P < 0.05$. Significantly greater than tamoxifen citrate without Arf; ***, $P < 0.001$.

μM) or PKC-α (100 nM). Although tamoxifen stimulates PLD1 through RhoA, the effects with PKC-α are inhibitory. By contrast, raloxifene inhibits PLD1 activity in all conditions, suggesting a different mechanism for a PLD interaction. To further illuminate the mechanism of the activation of PLD and possible sites of action by tamoxifen and raloxifene, we tested the activators RhoA and PKC-α on a partially purified amino-terminal truncation of PLD1, amino acids 325-1074 (Fig. 5B). Whereas the basal activity of truncated PLD1 is not stimulated by 20 μM tamoxifen, this stimulation is restored by RhoA, and unaffected in the presence of PKC-α. Stimulation by PKC-α is unremarkable in the truncated PLD1 basal condition as this site has previously been shown to be essential for PKC stimulation of PLD1 (Park et al., 1998; Sung et al., 1999). Addition of raloxifene (20 μM) to truncated PLD1 inhibits the basal activity, the stimulation by RhoA, and the basal activity in the presence of PKC-α. All

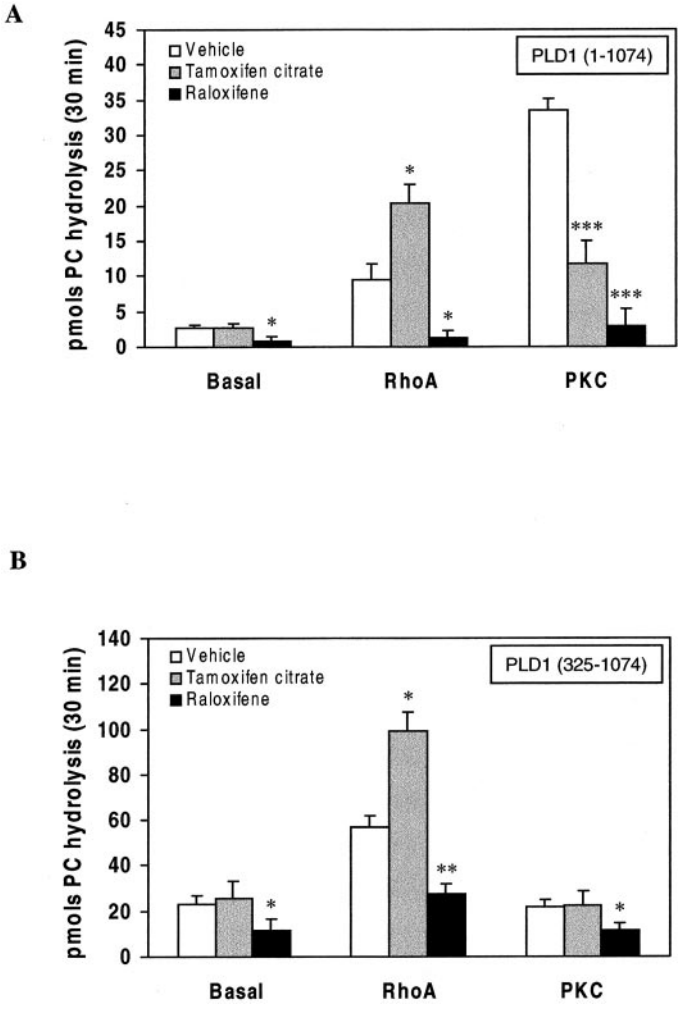

Fig. 5. Data are mean ± S.E. from four independent 30-min in vitro assays. Comparison of activities of 0.2 μg of full-length PLD1 (amino acids 1–1074) (A) or 7 μg of truncated PLD1 (amino acids 325-1074) (B) protein enriched by cation exchange chromatography in the presence of 20 μM tamoxifen citrate (▨), raloxifene (■), or vehicle (□). Note that raloxifene inhibits the basal and activator stimulated PLD1 activity in all conditions. Tamoxifen stimulates both full-length and truncated PLD1 constructs in the presence of the monomeric G-protein RhoA. However, addition of tamoxifen in the presence of PKC inhibits full-length PLD1 activity and has no effect on truncated PLD1 (325–1074). Significantly different from vehicle, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

PKC-α effects in Fig. 5, A and B, are the same for the PKC-βII isoform (data not shown).

To identify the precise role of the monomeric G-protein in the stimulation of PLD by tamoxifen, we explored the activities of various mutants of the well-characterized, PLD-stimulating G-protein Cdc42 (Walker and Brown, 2000, 2002) (Fig. 6). As predicted, wild-type Cdc42 can significantly restore the ability of tamoxifen to stimulate a purified preparation of PLD. To investigate whether the activity of tamoxifen was to accelerate GTP loading, and hence activation of the G-protein, we tested the GTP-bound, activated mutant of Cdc42, Q61L, and the GTP-bound fast-cycling mutant of Cdc42, F28L (Lin et al., 1997) (Fig. 6A). We found that both Q61L and F28L can significantly enhance PLD stimulation by tamoxifen over vehicle. In addition, we found that raloxifene not only inhibits basal PLD stimulation in the absence of Cdc42, but also inhibits the stimulation by the wild-type and activated mutants of Cdc42. These results strongly suggest that the stimulatory actions of tamoxifen and the inhibitory actions of raloxifene are not through guanine nucleotide exchange on the G-protein. To confirm this, we repeated these experiments on Q61L in the absence of additional guanosine 5′-*O*-(3-thio)triphosphate and found that native Q61L was able to restore the ability of tamoxifen to stimulate PLD (data not shown). Furthermore, we performed a GDP exchange assay to measure the dissociation of [^{3}H]GDP in the presence of tamoxifen and raloxifene and found no effect on the intrinsic exchange rate of Cdc42 in the presence of the SERMs (Fig. 6B). In Fig. 6C, we show the same in vitro assay as in Fig. 6A, using the dominant-negative mutant of Cdc42 T17N, and a mutant that can bind to but not activate PLD, Cdc42 S124A (Walker and Brown, 2002). Whereas raloxifene inhibits the basal PLD activity in all conditions, tamoxifen significantly stimulates PLD activity in the presence of S124A but not T17N. This suggests that the G-protein factor required to restore stimulation of PLD by tamoxifen is also required to bind to PLD and cause a conformational change to a transition state between the inactive ground state and a state of full catalytic activity. This further supports a mechanism of multiple transition states in the activation of PLD as described previously by Walker and Brown (2002). However, the inhibitory effects of raloxifene are maintained in the absence of a factor, suggesting a distinct, possibly direct interaction with PLD. Despite the pharmacological similarity of these compounds at the estrogen receptor, the mechanisms of action on PLD suggest that long-term consequences of signal transduction and the subsequent effects on cell membrane composition are profoundly different.

Discussion

We set out to determine whether SERMs such as tamoxifen and raloxifene have distinct consequences of signaling to PLD. Consistent with previous reports we found that tamoxifen stimulates PLD activity in ER-negative cells (Kiss, 1994). Recent studies have implicated the importance of PLD in transformation (Yoshida et al., 1998; Uchida et al., 1999; Fiucci et al., 2000; Noh et al., 2000; Zhao et al., 2000), so it is interesting to note that a widely used therapeutic like tamoxifen has effects on PLD. The differential effects on PLD by tamoxifen and raloxifene may contribute to observed differences in described side effects, specifically an increased risk

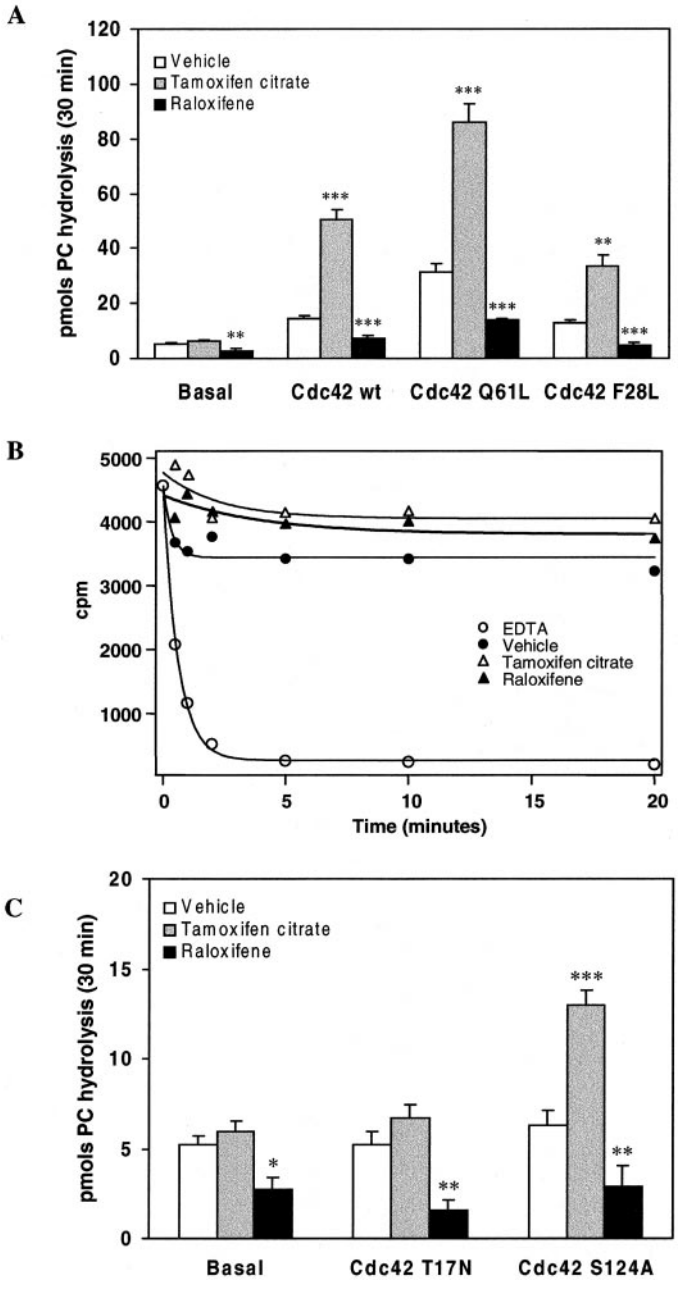

Fig. 6. Data are mean ± S.E. from four independent 30-min in vitro assays done in duplicate. Measurement of the activity of 0.2 μg partially purified PLD1 with cdc42 (1 μM) in the presence of 20 μM tamoxifen citrate (▨), raloxifene (■), or vehicle (□). A, tamoxifen stimulates PLD activity in the presence of wild-type (wt), activated (Q61L), and the fast-cycling mutant (F28L) of Cdc42, but not in the absence of Cdc42. Note that raloxifene inhibits the basal PLD activity in addition to the Cdc42 stimulated activity. B, measurement of the dissociation of [^{3}H]GDP from wild-type Cdc42 (1 μM) in the presence of 100 μM nonradioactive GDP and tamoxifen citrate (20 μM), raloxifene (20 μM), or vehicle. Graph represents mean of two independent 20-min experiments done in duplicate. Note that SERMs have no effect on [^{3}H]GDP dissociation. Curves were obtained using a single exponential fitting regime in the IGOR Pro software package (Wavemetrics, Lake Oswego, OR). C, measurement of the activity of 0.2 μg of partially purified PLD1 with Cdc42 (1 μM) in the presence of 20 μM tamoxifen citrate (▨), raloxifene (■), or vehicle (□). Tamoxifen does not stimulate PLD activity in the presence of dominant-negative (T17N) Cdc42, but does stimulate PLD with a mutant of Cdc42 (S124A) capable of binding, but not activating PLD. Note that raloxifene inhibits the basal PLD activities in all conditions. Significantly different from vehicle, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of endometrial cancer (Fisher et al., 1998) in patients undergoing prolonged tamoxifen treatment. The finding that raloxifene decreases PLD activity in vivo and in vitro, suggests the potential importance of determining the signaling mechanisms that regulate these distinct interactions. Considering the implications of PLD activities in the production of signaling molecules like PA in the growth and metastasis of cancers, it is highly desirable to study the mechanisms of action of a pharmacological compound that can significantly inhibit PLD enzymatic activity.

Our data provide evidence to support the differential effects of tamoxifen and raloxifene on the regulation of PLD. We demonstrate that the inhibitory effects of raloxifene are targeting PLD because raloxifene inhibits the basal PLD activity in MDA-MB-231 mammary carcinoma cells, endogenous PLD activity stimulated through three distinct pathways, and purified preparations of PLD1 in the absence of exogenously added activators. By contrast, PLD1 stimulation by tamoxifen requires the addition of a monomeric G-protein activator of PLD, but this single variable system can be reconstituted in vitro. The stimulatory effects of tamoxifen on PLD are not mediated by an interaction with PKC. In fact, the stimulatory effects of PKC on PLD1 are attenuated in the presence of tamoxifen. PLD1 stimulation is not regulated by the kinase domain of PKC (Park et al., 1998; Sung et al., 1999), and because tamoxifen binds not to this site on PKC (O'Brian et al., 1986) but to the regulatory domain (Bignon et al., 1991), we hypothesize that the decrease in PLD1 activity results from this tamoxifen binding. Tamoxifen would interfere with the ability of PKC to bind to and activate PLD1, suggesting a possible mechanism for this particular interaction and potentially one of some pharmacological relevance.

Interestingly, we discovered that the active metabolite of tamoxifen, 4-OH-tamoxifen, can distinguish the PLD1 and PLD2 isoforms. When measuring in vitro activity, we demonstrate that 4-OH-tamoxifen stimulates PLD1 but inhibits PLD2. This finding provides us with a pharmacological tool to help differentiate between the isoforms in vivo. Accordingly, our results suggest that the isoform of PLD that is hyperactive in the mammary carcinoma cell line MDA-MB-231 is PLD2. A Western blot analysis confirms the expression of PLD2 in these cells, and in vitro measurements of PLD activity in MDA-MB-231 cell lysates show 4-OH-tamoxifen decreases PLD activity (data not shown). Furthermore, chronic treatment of these cells with 4-OH-tamoxifen decreases cell growth, supporting a role for PLD in cell proliferation. These data are consistent with other findings that PA formation may participate in certain cancers, specifically the hyperactivation of the PLD2 isoform as reported elsewhere (Fiucci et al., 2000; Zhao et al., 2000).

To elucidate the region(s) of PLD1 involved in the interaction(s) with tamoxifen and raloxifene, we explored the effects of these compounds on an amino-terminal truncation of PLD1 (amino acids 325-1074). We demonstrate that the amino terminus is not required for either tamoxifen stimulation through the monomeric G-protein activators of PLD or the inhibition of PLD by raloxifene. Although the mechanisms of action of these therapeutics may be different, neither require the amino-terminal portion of PLD1. The specific mechanisms that mediate the effects of tamoxifen and raloxifene on the PLD isoforms are unknown, however these findings offer insights into the role of G-proteins in the stim-

ulatory activity of tamoxifen. Using activated forms of Cdc42, we have shown that tamoxifen does not enhance guanine nucleotide exchange and hence promote PLD activation by the G-protein. Furthermore, the use of a dominant negative, nucleotide-free mutant of Cdc42 suggests that tamoxifen-mediated stimulation of PLD requires the G-protein to bind to PLD and trigger a conformational change to a transition state capable of partial lipase activity as opposed to simply acting as a coupling factor between tamoxifen and PLD. This was shown using the wild-type Cdc42 in the presence of 10 μM GDP, where GDP-bound Cdc42 could not restore tamoxifen-stimulated PLD activity (data not shown). Additionally, data from the S124A mutant, a mutant of Cdc42 that has been shown to bind PLD but not stimulate catalytic activity, is capable of significantly restoring partial tamoxifen stimulated PLD activity. This suggests that the bound and activated conformations of PLD are distinct, and that tamoxifen requires PLD to be in a "bound" transition state to see significantly increased activity mediated by tamoxifen. However, this is not the case with raloxifene. The inhibitory effects of raloxifene are demonstrated in the absence of activators, suggesting a novel and possibly direct interaction with PLD. Despite the fact that these compounds are indistinguishable at the level of the receptor, we provide evidence supporting ER-independent, pharmacological differences on the regulation of PLD enzymatic activity.

In conclusion, we show that PLD catalytic activity is elevated in MDA-MB-231 cells, an ER-negative, tumorigenic mammary carcinoma line, relative to activity in the nontumorigenic mammary epithelial line, MCF-12A. This activity is modulated by SERMs independent of an estrogen receptor. We show that tamoxifen and raloxifene have differential effects on PC hydrolysis and suggest that chronic overproduction of phosphatidic acid in vivo may participate in certain adverse reactions associated with tamoxifen. Interestingly, we find that 4-OH-tamoxifen can be used to pharmacologically discriminate between the mammalian isoforms of PLD1 and PLD2. Furthermore, we chromatographically resolve a tamoxifen-stimulating factor from cytosolic preparations and find that this fraction is coincident with the Arf protein. A simple reconstitution shows that tamoxifen-stimulation of PLD can be achieved by addition of any of the monomeric G proteins, Arf, Rho, or Cdc42. This finding suggests that stimulation of PLD by tamoxifen requires a conformational change in the enzyme that is induced by GTPase binding. In contrast, addition of a classical isoform of protein kinase C will not mediate the tamoxifen stimulation of PLD. Interestingly, the inhibitory effects of raloxifene on PLD catalytic activity are observed even the absence of protein activators. As increasing evidence for a role of PLD in transformation and metastasis emerges, it becomes increasingly important to understand the potential roles of lipid signaling pathways in these processes and to further define the effects of anticancer therapeutic agents on the regulated production of phosphatidic acid.

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