Journal of Cellular Biochemistry 89:520–528 (2003)

Phospholipase C–Protein Kinase C Mediated Phospholipase D Activation Pathway Is Involved in Tamoxifen Induced Apoptosis

Soo-Jung Ahn,[2] Mee-Sup Yoon,[3] Shin Hyuk,[4] Wonshik Han,[1] Yong-Dal Yoon,[4] Joong-Soo Han,[3] and Dong-Young Noh[1,2]*

[1]Department of Surgery, College of Medicine, Seoul National University, Seoul 110-744, Korea
[2]Cancer Research Institute, College of Medicine, Seoul National University, Seoul 110-744, Korea
[3]Institute of Biomedical Science and Department of Biochemistry, College of Medicine, Hanyang University, Seoul 133-791, Korea
[4]Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 133-791, Korea

Abstract Tamoxifen (TAM) is the endocrine therapeutic agent the most widely used in the treatment of breast cancer, and it operates primarily through the induction of apoptosis. In this study, we attempted to elucidate the non-ER mediated mechanism behind TAM treatment, involving the phospholipase C–protein kinase C (PLC–PKC) mediated phospholipase D (PLD) activation pathway, using multimodality methods. In TAM treated MCF7 cells, the PLC and PLD protein and mRNA levels increased. Phosphatidylethanol (PEt) and diacylglycerol (DAG) generation also increased, showing increased activity of PLD and PLCγ1. Translocation of PKCα, from cytosol to membrane, was observed in TAM treated cells. By showing that both PKC and PLC inhibitors could reduce the effects of TAM-induced PLD activation, we confirmed the role of PKC and PLC as upstream regulators of PLD. Finally, we demonstrated that TAM treatment reduced the viability of MCF7 cells and brought about rapid cell death. From these results, we confirmed the hypothesis that TAM induces apoptosis in breast cancer cells, and that the signal transduction pathway, involving PLD, PLC, and PKC, constitutes one of the possible mechanisms underlying the non-ER mediated effects associated with TAM. J. Cell. Biochem. 89: 520–528, 2003.

Key words: tamoxifen (TAM); phospholipase C (PLC); phospholipase D (PLD); protein kinase C (PKC); apoptosis

Tamoxifen (TAM) is a synthetic non-steroidal anti-estrogen agent that is widely used for treating all stages of breast cancer and has been approved for the prevention of breast cancer in high-risk women. The observed efficacy of TAM has been attributed both to growth arrest and to the induction of apoptosis within the breast cancer cells, and the induction of apoptosis may constitute one of the primary mechanisms by which the anti-tumor effect of TAM [Budtz, 1999; Cameron et al., 2000] comes about.

Although the primary mechanism of action for TAM is believed to be through the inhibition of estrogen receptors (ER), research over the years has indicated that additional, non-ER-mediated mechanisms also exist [Perry et al., 1995; Kang et al., 1996] which can be activated by the use of clinically relevant concentrations of TAM. The possible underlying mechanisms are signaling pathways involving such factors as protein kinase C (PKC), calmodulin, transforming growth factor-β (TGF-β) and the protooncogene c-myc, ceramide, and MAP kinase [O'Brian et al., 1985; Butta et al., 1992; Hardcastle et al., 1995; Mandlekar and Kong, 2001].

The stimulation of phospholipase D (PLD) occurs in a wide variety of cells treated with hormones and growth factors. PLD catalyzes the hydrolysis of phospholipids at their terminal phosphodiester bond, thus producing phosphatidic acid (PA) and releasing the free polar head group [Exton, 1990]. PA can serve as a messenger molecule with the potential to

Grant sponsor: Korea Science and Engineering Foundation (KOSEF); Grant number: 2000-2-20900-003-3.

*Correspondence to: Dong-Young Noh, Department of Surgery, College of Medicine, Seoul National University, 28 Yongon-Dong, Jongno-Gu, Seoul 110-744, Korea.
E-mail: dynoh@plaza.snu.ac.kr

Received 13 December 2002; Accepted 5 March 2003
DOI 10.1002/jcb.10532

regulate cell growth [Moolenaar et al., 1986], the metastasis of tumor cells [Imamura et al., 1993], exocytosis [Kinsky et al., 1989], as well as other cellular processes [Kiss, 1990]. PA can be further metabolized by a PA phosphohydrolase to form diacylglycerol (DAG), a PKC activator. Most mammalian PLD activity is found to be associated with membranes and appears to be specific for phosphatidylcholine (PC) [Exton, 1998]. Kiss [1994] proved that TAM can stimulate PLD activity by an estrogen receptor-independent mechanism.

PKC, a Ca^{2+}- and phospholipids-dependent enzyme, represents a large family of several isoenzymes that play a key role in the transmembrane signaling of a wide variety of stimuli, including growth factors. PKC has been implicated in the regulation of cellular growth and differentiation [Nishizuka, 1995]. The exact role of PKC in TAM-induced apoptosis is not clear and there is some controversy as to whether TAM activates or inhibits PKC [Horgan et al., 1986; Cheng et al., 1998]. It has been demonstrated that TAM induces the membrane translocation of PKC, and this translocation is regarded as a key process in the TAM-induced apoptosis pathway [Gundimeda et al., 1996; Lavie et al., 1998].

We proposed a model of the phospholipase C–phospholipase D (PLC–PLD) crosstalk-mediated TAM-induced apoptosis-signaling pathway, which is shown in Figure 7. PLCγ1 might be activated by extracellular stimuli such as TAM, subsequently generating two-second messengers, DAG and IP3, as a result of its PIP2 (phosphatidylinositol 4,5 bisphosphate) hydrolyzing activity. DAG and IP3 induce a host of intracellular reactions that eventually give rise to various cellular processes. DAG is a well-known activator of PKC. PLD activation is mediated by activated PKCα, resulting from its translocation from cytosol to membranes in the cell. Activation of PLD may contribute to the transcriptional induction of death-related genes or they may cross-communicate with the effectors of apoptosis.

MATERIALS AND METHODS

Materials

Phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, Triton X-100, DTT, HEPS, and diethylpyrocarbonate (DEPC) were obtained from Sigma Aldrich (St. Louis, MO). TRIzol reagent was obtained from Invirogen Life Technologies (Carlsbad, CA). The First strand cDNA synthesis kit was obtained from Roche Applied Science (Mannheim). *Taq* DNA polymerase was obtained from TaKaRa Biotechnology Inc. (Japan). Protein G Sepharose was obtained from Amersham Pharmacia Biotech (Piscataway, NJ) products. Peroxidase conjugated goat anti-rabbit IgG antibody was obtained from Jackson Immuno Research Laboratories, Inc. (Pennsylvania). The Enhanced Chemiluminescence Detection (ECL) system, [^{3}H] palmitic acid (154.0 Ci/mmol) and [methyl-^{3}H] choline chloride (83.0 Ci/mmol) were from Amersham Pharmacia Biotech. Silica Gel 60 A thin-layer chromatography plates were purchased from Whatman (Wiesioch). Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL). The protein concentration was determined using a protein assay kit from Pierce Biotechnology (Rockford, IL).

Cell Culture, Treatment, and Viability Assays

The human mammary carcinoma cell line MCF7 was obtained from the Korean Cell Line Bank and maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (100 U/ml) in a 37°C incubator under 5% CO_2.

For the cell viability assays, cells were plated in 60-mm dishes and cultured overnight prior to treatment with TAM. Following treatment, the cells were harvested, stained with trypan blue, and live cells were counted using a hemocytometer.

Annexin V-FITO Apoptosis Detection

Both TAM treated and control MCF7 cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. In each case, 100 μl of the solution was transferred to a 5-ml culture tube, to which was added 5 μl of annexin V-FITC and 5 μl of PI. After being gently vortexed, the cells were incubated for 15 min at room temperature in the dark. Finally, 400 μl of 1× binding buffer was added to each tube, an analysis was performed by flow cytometry within 1 h.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the TAM treated MCF7 cells using TRIzol reagent. The RNA

pellet was dissolved in DEPC-treated H_2O to concentrations of approximately 0.5–1.0 μg/μl, and stored at −70°C. The quantity and quality of the RNA preparations were determined by absorbance at 260 and 280 nm. One microgram of total RNA sample was reverse transcribed using the first strand cDNA synthesis kit with random primer $p(dN)_6$, according to the manufacturer's instructions. RT-PCR analysis was performed. Primers used for amplification were synthesized as follows: *h*PLD1; forward primer nucleotides 1,475–1,491: 5′-TGGGCTCACCATGAGAA-3′; reverse primers nucleotides 2,133–2,113: 5′-GTCATGCCAGGGCATCCGGGG-3′. Amplification conditions used were 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min; 30 cycles.

Immunoblot Analysis

TAM treated MCF7 cells were homogenized in 20 mM HEPES (pH 7.2), 200 mM sodium chloride, 1% Triton X-100, 2% cholic acid, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Protein content was determined with bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard.

For the immunoblot analysis, the immunoprecipitates were washed five times with 1 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), and 200 mM sodium chloride. Immune complexes were released by heating the sample at 95°C for 10 min with Laemmli cooking buffer and separated by 8% SDS–polyacrylaminde gel electrophoresis (PAGE). The samples were transferred onto nitrocellulose filters and incubated separately with their respective monoclonal antibodies for 2 h. Immunoreactive bands were visualized using peroxidase conjugated goat anti-rabbit IgG antibody. Detection was performed with the ECL system.

In Vivo PLD Enzyme Activity Assay, DAG Generation Assay

Exponentially growing cells were transferred into 6-well plates (1.5×10^6 cells/well) and then incubated for 24 h so that the cells stuck on the culture dishes. For isotope labeling, an exchange of media was operated with RPMI 1640 media containing 1% FBS and 1 μCi/ml of (^{3}H) palmitic acid, and then the cells were incubated for 24 h. After washing with PBS twice, the cells were preincubated for 1 h in a warmed serum-free medium. The cells were pretreated with 1% (v/v) ethanol for 10 min before the treatment of 1 μM TAM. After the stimulation of TAM (0, 5, 10, 30, 60, and 180 min), the cells were quickly washed with ice-cold PBS and suspended in 0.5 ml of ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer [1959].

DAG was separated from other phospholipids by TLC using a solvent system consisting of toluene/ether/ethanol/NH_4OH (50/30/2/0.2, v/v/v/v). The regions corresponding to the standard were identified with primulin. Then DAG regions were scraped and the radioactivity counted using an LSC.

Phosphatidylethanol (PEt) was separated from other phospholipids by TLC using a solvent system consisting of ethyl acetate/isooctane/acetic acid/water (130/20/30/100, v/v/v/v). In this solvent system, water was used for the saturation of the organic solvent. Thus, after mixing all of contents well, water was separated from the upper organic phase and eliminated. The regions corresponding to the standard were identified with primulin. Then, the PEt regions were scraped and the radioactivity counted using an LSC.

Data are represented as mean ± SEM from three separate experiments.

Quantification of Autoradiographs

An Biomedlab computing densitometer was used to digitize images. The optical density of bands was analyzed with background subtraction using ImageQuant software.

RESULTS

The first goal was to determine the possible role of PLD1 and PLCγ1 in time-dependent 1 μM TAM treated MCF7. Using degenerated primers, based on the conserved amino acid regions, we amplified *h*PLD1 in time-dependent 1 μM TAM treated MCF7 by means of the RT-PCR. The pattern corresponding to the expression level of PLD1 mRNA in MCF7 increased with increasing TAM treatment time (Fig. 1A,B). We performed immunoblot analysis to demonstrate that PLD production also increased as the transcription level increased. For normalization of RT-PCR and immunoblot analysis, β-actin was amplified simultaneously.

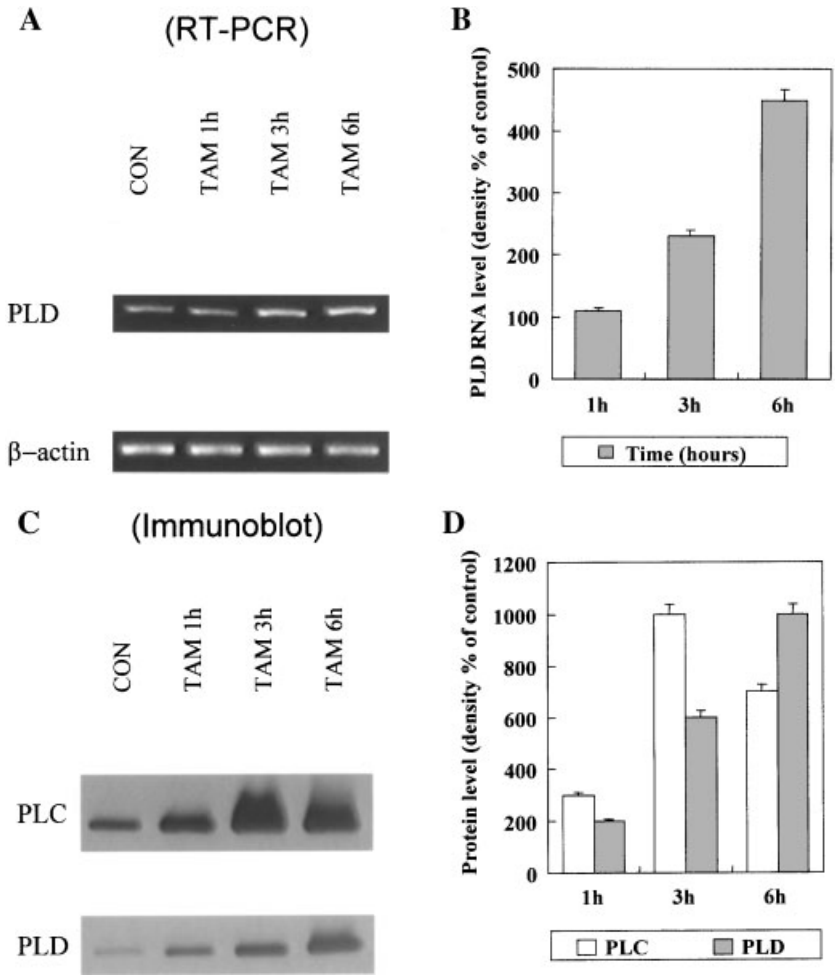

Fig. 1. The effect of tamoxifen (TAM) on the expression levels of protein kinase C (PLC) and phospholipase D (PLD). **A**: The cells were serum-starved for 24 h in 100 mm culture dishes, and stimulated with 1 μM TAM for 1, 3, and 6 h. The total RNA was isolated with TRIzol reagent. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was done as described in Materials and Methods. Combined PCR products were resolved by 2% agarose gel. Bands were visualized following EtBr staining and were quantified using ImageQuant software. **B**: Densitometry measurements from three time course experiments of 1 μM TAM and control cells demonstrating increased PLD RNA levels (data presented as mean ± SE, $P < 0.05$ compared with control value). **C**: The cells were homogenized and total lysates were obtained. Twenty micrograms of lysates were analyzed by 10% sodium dodecylsulfate–polyacrylaminde gel electrophoresis (SDS–PAGE), and transferred to nitrocellulose membrane. Western blotting using anti-PLC and anti-PLD antibodies, as described in Materials and Methods, was used to identify the proteins and was quantified using ImageQuant software. **D**: Densitometry measurements from three time course experiments demonstrating a time-related increase in PLC and PLD protein levels (data presented as mean ± SE, $P < 0.001$ compared with control value).

The level of PLD1 and PLCγ1 in 1 μM TAM treated MCF7 showed an apparent time-dependent increase (Fig. 1C,D). As shown in these two results, the amplification of PLD1 mRNA expression, analyzed by RT-PCR, was well correlated with the result of the immunoblot analysis.

In addition, we measured in vivo PLD activity and performed DAG generation assay in time-dependent 1 μM TAM treated MCF7 cells. The [^{3}H] palmitic acid-labeled and serum-starved MCF7 cells were treated with 1 μM TAM at various moments in time. After being harvested, cells were assayed by TLC. To determine whether TAM activates cellular PLD, we added ethanol to the medium as this enables the formation of PEt, the transphosphatidylation product of PLD, at the expense of PA. As shown in Figure 2A, the production of PEt increased gradually as the TAM treatment time increased from 10 min to 3 h. This data showed that PLD activity increased, reaching a maximum of about eightfold, at 3 h after stimulation by TAM. Concurrent with this increase in the

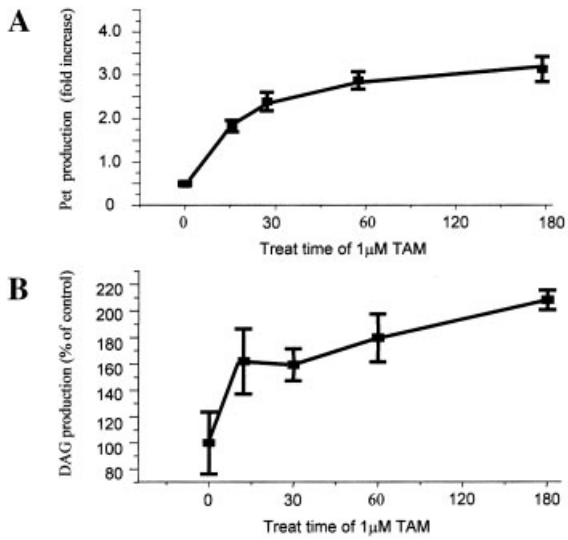

Fig. 2. PLD activity and diacylglycerol (DAG) production in time-dependent TAM treated MCF7 cell lines. Measurement of PLD activity and DAG production after stimulation of TAM (0, 30 min; 1, 3, and 6 h) in MCF7 cells was performed using the PLD activity assays (**A**) and DAG production (**B**) as described in Materials and Methods. Data are represented as mean ± SEM from three separate experiments.

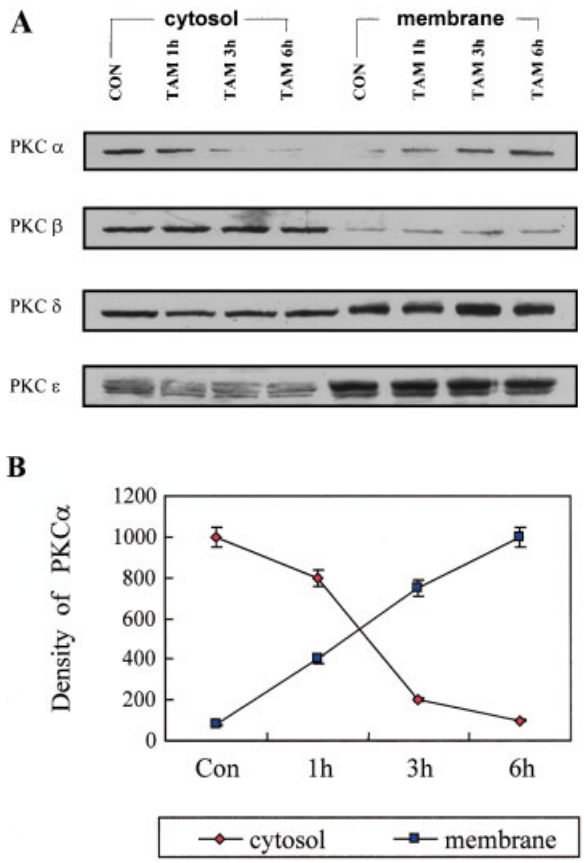

Fig. 3. Translocation of protein kinase C (PKC) in TAM treated MCF7. **A**: The cells were serum-starved for 24 h in 100 mm culture dishes, and stimulated with 1 μM TAM for 1, 3, and 6 h. The cells were homogenized and total lysates obtained. Twenty grams of lysates were analyzed by 12% SDS–PAGE, and transferred to nitrocellulose membrane. Western blotting using anti-PKC isoforms antibodies, as described in Materials and Methods, was used to identify the proteins and was quantified using ImageQuant software. **B**: Densitometry measurements from three time course experiments demonstrating time-related translocation in PKCα protein levels (data presented as mean ± SE, $P < 0.001$ compared with control value). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

amount of PEt, the DAG production rate increased up to 65% of its maximum level within 10 min after treatment with 1 μM TAM (Fig. 2B). These results showed that the initial increase in PEt production resulted from increasing PLD activity, and that the increased DAG production rate resulted from an increase in PIP2 hydrolysis caused by TAM-induced PLCγ1.

A major pathway for PLD activation by extracellular signals involves PKC translocation and activation. In Figure 3A,B, we demonstrated the translocation of PKCα from cytosol to membrane in 1 μM TAM treated MCF7 cells. Other isozymes tested were not responsive to TAM treatment. This result conforms to the hypothesis that TAM-induced activation of PLD is mediated by PKCα translocation.

For further confirmation of the hypothesis that PKC is the upstream of TAM-induced PLD activation, we demonstrated that the effect of TAM on PLD activity changed, following the desensitization of PKC by long-term stimulation with PMA. The PEt assay revealed that the PEt contents of cells, desensitized with PMA, decreased by about 40% after 30 min of TAM stimulation, compared with those of undesensitized TAM treated cells (Fig. 4).

We performed an additional experiment to establish the role of PKC in PLD activation. As shown in Figure 5B, the maximum level of TAM-induced PLD activity was lowered by about 25% when we added GF109202X, the PKC inhibitor.

We observed the change in TAM-induced PLD activity after treatment with the PLC inhibitor. The PEt assay revealed that the PEt contents of PLC inhibited cells with D609 decreased by about 40% and that the PEt contents of PLC inhibited cells with U73122 decreased by about 65% compared with the maximum PLD activity resulting from TAM stimulation alone (Fig. 5A). Figure 5C,D showed that the effect of TAM on PLC expression was

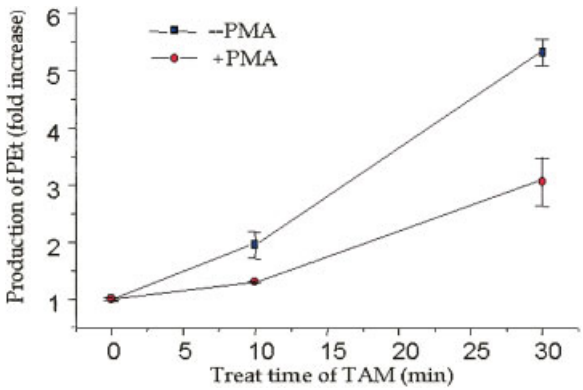

Fig. 4. Effect of TAM on PLD activation after desensitization of PKC. The cells were incubated with (●) or without (■) 200 nM PMA for 24 h, at the same time the cells were labeled with [^{3}H] palmitic acid and serum-starved. Measurement of PLD activity after the stimulation of TAM (0, 10, 30 min) in MCF7 cells was performed using the PLD activity assays as described in Materials and Methods. Data are represented as mean ± SEM from three separate experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

negated following PLC inhibition by treatment with D609 and U73122.

It is well known that DAG originates from three different pathways. The first one is from PC hydrolysis by PC–PLC enzyme activation, the second one is from PIP2 hydrolysis by PI–PLC and the third one is from the phosphohydrolysis of PA by PLD.

We demonstrated that U73122, which acts as an inhibitor of PI–PLC, rather than D609, which is an inhibitor of PC–PLC, was more effective in inhibiting TAM-induced PLD activity (Fig. 5A) and PLC expression (Fig. 5C,D). These findings suggest that DAG, as a product of PI–PLC activation, participates in TAM-induced PLD activation.

Finally, we performed cell viability assay (Fig. 6A,B) and annexin V apoptosis assay (Fig. 6C), to investigate TAM-induced cell death in MCF7. In cell viability assay, we showed the time dependent reduction of cell viability in 1 μM TAM treated MCF7 cells compared to the control (Fig. 6A).

To establish a link between the regulation of PLC/PLD mechanism and the induction of cell death by TAM, we performed cell viability and annexin V apoptosis assay after treatment with PLC inhibitors. As shown in Figure 6B,C showed that PLC inhibitors treated MCF7 cells inhibit TAM-induced cell death. In cell viability assay, we showed decrease cell viability and in annexin V assay, the 23% increase in the proportion of cell death in 1μM TAM treated

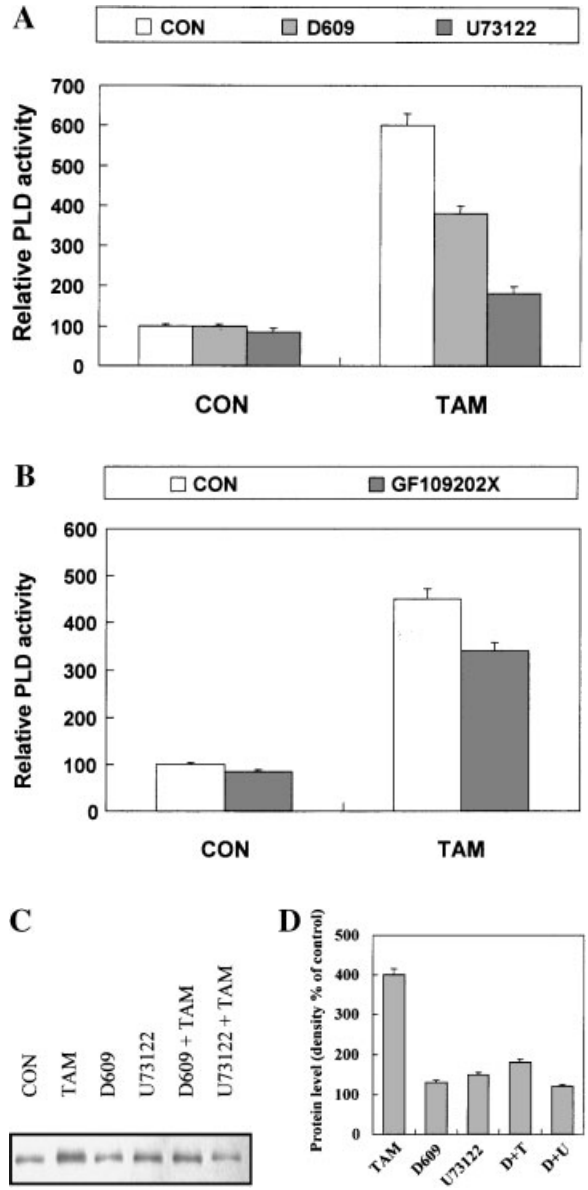

Fig. 5. Effects of PLC and PKC inhibitors on TAM mediated PLD activiry. The cells were pre-incubated with (**A**) D609, U73122, and (**B**) GF109202X for 30 min, after being labeled with [^{3}H] palmitic acid and serum-starved for 24 h. Measurement of PLD activity after the stimulation of TAM in MCF7 cells was performed using the PLD activity assays as described in Materials and Methods. Data are represented as mean ± SEM from three separate experiments. **C**: The cells were serum-starved for 24 h in 100 mm culture dishes, pre-incubated with D609 and U73122 for 30 min, and stimulated with 1 μM TAM for 6 h. The cells were homogenized and total lysates obtained. Twenty micrograms of lysates were analyzed by 10% SDS–PAGE, and transferred to a nitrocellulose membrane. Western blotting using anti-PLC antibodies as described in Materials and Methods was used to identify the proteins and was quantified using ImageQuant software. **D**: Densitometry measurements from three experiments demonstrating that D609 and U73122 inhibited TAM-induced PLC protein production in MCF7 cells (data presented as mean ± SE, $P < 0.05$ for TAM-treated cells versus TAM plus D609, U73122-treated cells).

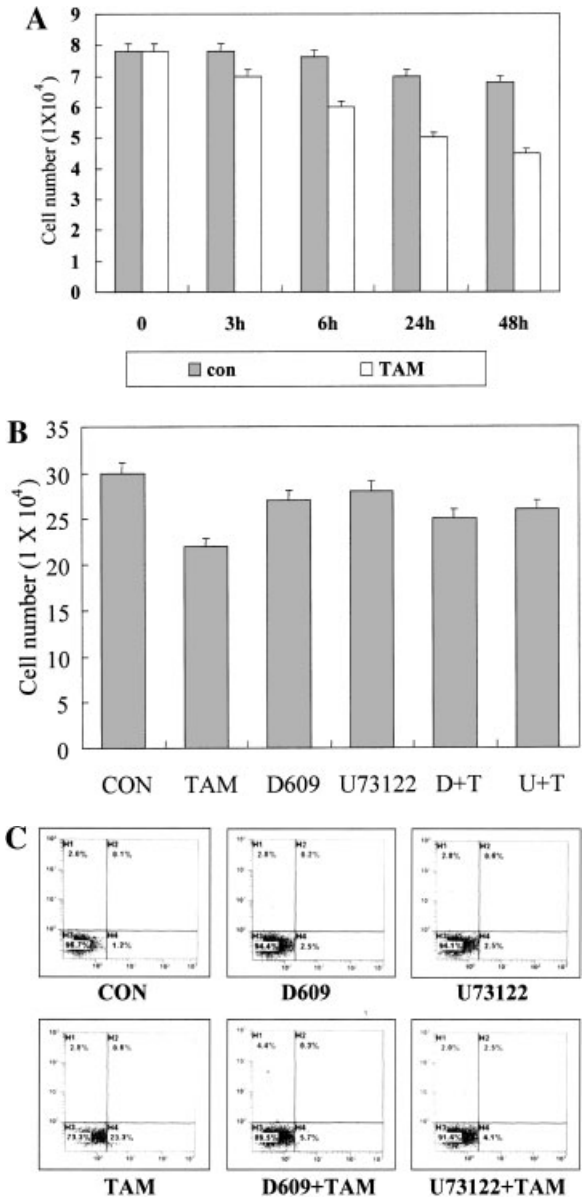

Fig. 6. Cell viability and apoptosis in TAM treated MCF7. **A**: The cells used for the viability assay were serum-starved for 24 h in 6-well culture plates and stimulated with 1 μM TAM for 0, 3, 6, 24, and 48 h. The cells were harvested, stained with trypan blue, and the number of live cells was counted using a hemocytometer. **B**: The cells used for the viability assay were serum-starved for 24 h in 6-well culture plates and pre-incubated with D609 and U73122 for 30 min and stimulated with 1 μM TAM for 24 h. The cells were harvested, stained with trypan blue, and the number of live cells was counted using a hemocytometer. **C**: The cells used for the apoptosis assay were serum-starved for 24 h in 6-well culture plate and pre-incubated with D609 and U73122 for 30 min and stimulated with 1 μM TAM for 24 h. TAM treated and control MCF7 cells were washed twice with cold PBS and then resuspended in 100 μl of 1× binding buffer. Five microliters of annexin V-FITC and 5 μl of PI were added and the plates were incubated for 15 min at room temperature in the dark. 400 μl of 1× binding buffer was added to each tube. Analysis was performed by flow cytometry.

MCF7 cells. In construct, we showed that cell death inhibits TAM treatment after pre-treat PLC inhibitors or only treatment PLC inhibitors in MCF7 cells (Fig. 6C).

DISCUSSION

There is much evidence that PKC is a major mediator of agonist action upon PLD. It has been known for some time that treatment of many cell types, with agonists that induce PIP2 hydrolysis or with phorbol esters, stimulates PLD activity, which implies the regulation of the enzyme by PKC [Kiss, 1996]. Furthermore, the treatment of cells with PKC inhibitors usually leads to the inhibition of the agonist stimulation of PLD. Likewise, the down-regulation of PKC, by prolonged treatment with phorbol ester, generally leads to a loss of PLD response to agonists [Kiss, 1996].

The mechanism by which PKC regulates PLD in intact cells is unclear. The stimulatory effect of the PKC isoenzymes does not require ATP [Min et al., 1998] and is not associated with protein phosphorylation [Singer et al., 1996]. Thus, the activation of PLD seems to occur through a non-phophorylating protein–protein interaction. The failure of the Ca^{2+} dependent γ- and other PKC isoenzymes to activate PLD indicates that regulatory domain sequences, unique to the α- and β-isozymes, are involved in the interaction [Conricode et al., 1994; Min et al., 1998].

The generally accepted mechanism, by which agonists activate PKC, is through the activation of PLC isoenzymes, which hydrolyze PIP2 to release IP3 and DAG [Exton, 1998]. The absolute dependence of growth factor-induced PLD activation upon PLC activity was demonstrated in two previous studies [Yeo et al., 1994; Hess et al., 1998].

It has been shown that TAM induces apoptosis both in vitro and in vivo [Perry et al., 1995; Martin et al., 1996]. According to the in vitro studies, this effect was concentration dependent. At nanomole concentrations of TAM, only growth arrest occurs, whereas at micromole concentrations induction of cell death was observed in cell cultures. We used 1 μM TAM in our study, and we were able to induce apoptosis.

There is some controversy about the antagonist/agonist action of TAM on PKC. Earlier studies demonstrated that TAM inhibited PKC [O'Brian et al., 1985; Horgan et al., 1986;

Issandou et al., 1990] and proposed that such action was central to its antitumor activity. In one instance, TAM did not appear to inhibit the catalytic action of PKC but reduced the binding of phorbol dibutyrate in a cell-free assay [O'Brian et al., 1985]. In another example, using intact neutrophils, TAM inhibited phorbol ester-stimulated oxidase activation [Horgan et al., 1986]. But in more recent studies, PKC activation by triphenyl-ethylenes has been demonstrated in vitro and in intact cells [Bignon et al., 1991]. TAM can be inhibitory for PKC when assayed in cell-free systems [Horgan et al., 1986], but has an opposite influence in intact cells [Issandou et al., 1990]. The activation of PKC is usually associated with a mitogenic response. However, in human breast cancer cells it has been amply demonstrated that TPA and DAG, activators of PKC, inhibited cell growth [Issandou et al., 1990]. Lavie et al. [1998] and Cabot et al. [1997] showed that TAM elicited both the isoenzyme-specific membrane translocation of PKC-ε and the inhibition of cell growth.

There have been many studies on the upstream of PKC in the TAM-induced apoptosis pathway. Direct binding of TAM to the triphenylethylene binding site on PKC may modulate its activity [O'Brian et al., 1988] or this may be mediated through PLC [Cabot et al., 1997] or oxidative stress [Gundimeda et al., 1996] or proteolytic activation by caspase [Datta et al., 1997]. But, Eisen and Brown [2002] reported that stimulation of PLD by TAM requires a conformational change in the enzyme that is induced by GTPase binding. In contrast, addition of a classical isoform of PKCα not mediates the TAM stimulation of PLD.

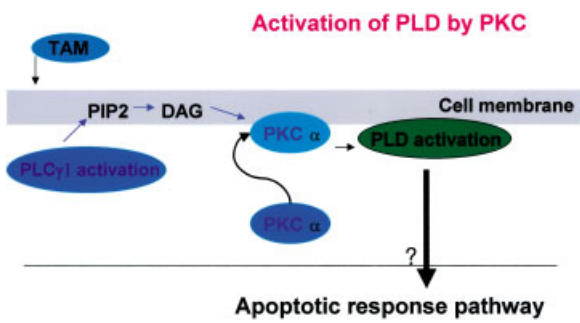

Fig. 7. Model of the PLC–PKC–PLD mediated TAM-induced apoptosis signaling pathway. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Induction of apoptosis has been regarded as a primary mechanism underlying the observed anti-tumor effects of TAM. Efforts to elucidate the non-ER mediated, non-genomic mechanism of TAM have been made on both the cellular and molecular levels. Various discrete signaling intermediates have been reported to play a role in the pro-apoptic mechanisms of TAM. However, no single signal transduction pathway has been unequivocally shown to constitute the underlying apoptotic mechanism elicited by TAM [Budtz, 1999]. In this study, we proposed a model involving PLC, PLD, and PKC as being one possible such mechanism (Fig. 7). Although we were able to demonstrate some essential components of this pathway, further investigation will be required, in order to fill in the missing links, and to demonstrate the relevance of this signaling pathway in the clinical setting.

REFERENCES

Bignon E, Pons M, Dore JC, Gilbert J, Ojasoo T, Miquel JF, Raynaud JP, Crastes de Paulet A. 1991. Influence of di- and tri-phenylethylene estrogen/antiestrogen structure on the mechanisms of protein kinase C inhibition and activation as revealed by a multivariate analysis. Biochem Pharmacol 42:1373–1383.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–919.

Budtz PE. 1999. Role of proliferation and apoptosis in net growth rates of human breast cancer cells (MCF-7) treated with oestradiol and/or tamoxifen. Cell Prolif 32: 289–302.

Butta A, MacLennan K, Flanders KC, Sacks NP, Smith I, McKinna A, Dowsett M, Wakefield LM, Sporn MB, Baum M. 1992. Induction of transforming growth factor beta 1 in human breast cancer in vivo following tamoxifen treatment. Cancer Res 52:4261–4264.

Cabot MC, Zhang Z, Cao H, Lavie Y, Giuliano AE, Han TY, Jones RC. 1997. Tamoxifen activates cellular phospholipase C and D and elicits protein kinase C translocation. Int J Cancer 70:567–574.

Cameron DA, Keen JC, Dixon JM, Bellamy C, Hanby A, Anderson TJ, Miller WR. 2000. Effective tamoxifen therapy of breast cancer involves both antiproliferative and pro-apoptotic changes. Eur J Cancer 36:845–851.

Cheng AL, Chuang SE, Fine RL, Yeh KH, Liao CM, Lay JD, Chen DS. 1998. Inhibition of the membrane translocation and activation of protein kinase C, and potentiation of doxorubicin-induced apoptosis of hepatocellular carcinoma cells by tamoxifen. Biochem Pharmacol 55:523–531.

Conricode KM, Smith JL, Burns DJ, Exton JH. 1994. Phospholipase D activation in fibroblast membranes by the alpha and beta isoforms of protein kinase C. FEBS Lett 342:149–153.

Datta R, Kojima H, Yoshida K, Kufe D. 1997. Caspase-3-mediated cleavage of protein kinase C theta in induction of apoptosis. J Biol Chem 272:20317–20320.

Eisen SF, Brown HA. 2002. Selective estrogen receptor (ER) modulators differentially regulate phospholipase D catalytic activity in ER-negative breast cancer cells. Mol Pharmacol 62(4):911–920.

Exton JH. 1990. Signaling through phosphatidylcholine breakdown. J Biol Chem 265:1–4.

Exton JH. 1998. Phospholipase D. Biochim Biophys Acta 1436:105–115.

Gundimeda U, Chen ZH, Gopalakrishna R. 1996. Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells. J Biol Chem 271:13504–13514.

Hardcastle IR, Rowlands MG, Houghton J, Parr IB, Potter GA, Jarman M, Edwards KJ, Laughton CA, Trent JO, Neidle S. 1995. Rationally designed analogues of tamoxifen with improved calmodulin antagonism. J Med Chem 38:241–248.

Hess JA, Ji QS, Carpenter G, Exton JH. 1998. Analysis of platelet-derived growth factor-induced phospholipase D activation in mouse embryo fibroblasts lacking phospholipase C-gamma1. J Biol Chem 273:20517–20524.

Horgan K, Cooke E, Hallett MB, Mansel RE. 1986. Inhibition of protein kinase C mediated signal transduction by tamoxifen. Importance for antitumour activity. Biochem Pharmacol 35:4463–4465.

Imamura F, Horai T, Mukai M, Shinkai K, Sawada M, Akedo H. 1993. Induction of in vitro tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. Biochem Biophys Res Commun 193:497–503.

Issandou M, Faucher C, Bayard F, Darbon JM. 1990. Opposite effects of tamoxifen on in vitro protein kinase C activity and endogenous protein phosphorylation in intact MCF-7 cells. Cancer Res 50:5845–5850.

Kang Y, Cortina R, Perry RR. 1996. Role of c-myc in tamoxifen-induced apoptosis estrogen-independent breast cancer cells. J Natl Cancer Inst 88:279–284.

Kinsky SC, Loader JE, Benedict SH. 1989. Phorbol ester activation of phospholipase D in human monocytes but not peripheral blood lymphocytes. Biochem Biophys Res Commun 162:788–793.

Kiss Z. 1990. Effects of phorbol ester on phospholipid metabolism. Prog Lipid Res 29:141–166.

Kiss Z. 1994. Tamoxifen stimulates phospholipase D activity by an estrogen receptor-independent mechanism. FEBS Lett 355:173–177.

Kiss Z. 1996. Regulation of phospholipase D by protein kinase C. Chem Phys Lipids 80:81–102.

Lavie Y, Zhang ZC, Cao HT, Han TY, Jones RC, Liu YY, Jarman M, Hardcastle IR, Giuliano AE, Cabot MC. 1998. Tamoxifen induces selective membrane association of protein kinase C epsilon in MCF-7 human breast cancer cells. Int J Cancer 77:928–932.

Mandlekar S, Kong AN. 2001. Mechanisms of tamoxifen-induced apoptosis. Apoptosis 6:469–477.

Martin G, Melito G, Rivera E, Levin E, Davio C, Cricco G, Andrade N, Caro R, Bergoc R. 1996. Effect of tamoxifen on intraperitoneal *N*-nitroso-*N*-methylurea induced tumors. Cancer Lett 100:227–234.

Min DS, Park SK, Exton JH. 1998. Characterization of a rat brain phospholipase D isozyme. J Biol Chem 273: 7044–7051.

Moolenaar WH, Kruijer W, Tilly BC, Verlaan I, Bierman AJ, de Laat SW. 1986. Growth factor-like action of phosphatidic acid. Nature 323:171–173.

Nishizuka Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 9:484–496.

O'Brian CA, Liskamp RM, Solomon DH, Weinstein IB. 1985. Inhibition of protein kinase C by tamoxifen. Cancer Res 45:2462–2465.

O'Brian CA, Ward NE, Anderson BW. 1988. Role of specific interactions between protein kinase C and triphenylethylenes in inhibition of the enzyme. J Natl Cancer Inst 80:1628–1633.

Perry RR, Kang Y, Greaves B. 1995. Effects of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells. Ann Surg Oncol 2:238–245.

Singer WD, Brown HA, Jiang X, Sternweis PC. 1996. Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase activity. J Biol Chem 271:4504–4510.

Yeo EJ, Kazlauskas A, Exton JH. 1994. Activation of phospholipase C-gamma is necessary for stimulation of phospholipase D by platelet-derived growth factor. J Biol Chem 269:27823–27826.