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TAMOXIFEN ACTIVATES CELLULAR PHOSPHOLIPASE C AND D AND ELICITS PROTEIN KINASE C TRANSLOCATION

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The antiestrogen tamoxifen is widely used for endocrine therapy of breast cancer; however, the mechanisms of estrogen receptor-independent interactions of tamoxifen remain ill defined. Here we examine the effect of tamoxifen on the initial steps of cell signal transduction. To this end, phospholipid metabolism and protein kinase C (PKC) translocation were assessed in CCD986SK human mammary fibroblasts treated with tamoxifen. The addition of tamoxifen resulted in dose-dependent and time-dependent increases in the cellular second messengers phosphatidate (PA) and diacylglycerol (DG). On addition of ethanol to the medium, tamoxifen induced the formation of phosphatidylethanol, demonstrating that tamoxifen activates phospholipase D (PLD). Cellular DG also increased in the presence of ethanol, showing that tamoxifen also activates phospholipase C (PLC). In cells prelabeled with choline and ethanolamine, tamoxifen caused increases in choline, phosphorylcholine, ethanolamine and phosphorylethanolamine. Structure-activity relationship studies for activation of PLD revealed that tamoxifen was the most effective, whereas 4-*hydroxy* tamoxifen was nearly devoid of activity. Phorbol diesters also activated PLD, but estrogen had no influence. Pretreatment of cells with phorbol dibutyrate (PKC down-regulation protocol) blocked phorbol diester- and tamoxifen-induced PLD activity. Exposure of cells to the PKC inhibitor GF 109203X diminished tamoxifen-induced PLD activity. Addition of tamoxifen to cultures elicited selective membrane association of PKC ε. We conclude that tamoxifen exerts considerable extra-nuclear influence at the transmembrane signaling level. These events may contribute to effects beyond the scope of estrogen receptor-dependent actions. *Int. J. Cancer,* 70:567–574, 1997.
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Since its introduction in the early 1970s tamoxifen, a nonsteroidal anti-estrogen, has been widely used in the treatment of breast cancer (Lerner and Jordan, 1990). The potential of tamoxifen for preventing breast cancer is currently being evaluated in a nationwide trial involving more than 16,000 healthy women. Concern has, however, been expressed regarding the apparent estrogenic properties of tamoxifen for endometrium. This observation signals the need for more intensive studies on tissue-specific actions of tamoxifen and related anti-estrogen-based agents.

As is the case with tumors of glandular origin, many are growth stimulated by endocrine mediators. That the efficacy of tamoxifen lies in its ability to antagonize the action of estrogen by binding competitively to the estrogen receptor cannot be disputed. However, it has become increasingly apparent that tamoxifen has a number of other potentially significant interactions, sharing a role as an effective therapeutic agent in non-breast-related cancers. Tamoxifen use has been initiated for treatment of brain cancer (Couldwell *et al.,* 1993), and it is an important component of the Dartmouth regimen for advanced melanoma (Del Prete *et al.,* 1984). As a biological response modifier, tamoxifen treatment causes induction of transforming growth factor-β in human breast cancer (Butta *et al.,* 1992), inhibition of protein kinase C (PKC) (O'Brian *et al.,* 1985) and antagonism of calmodulin (Hardcastle *et al.,* 1995). With these diverse actions it is reasonable to suggest that tamoxifen elicits various responses via non-genomic pathways. Elucidation of these pathways may be key to understanding the multimodal effects of tamoxifen and may lead the way for new approaches to anti-estrogen drug design.

The work presented here demonstrates that tamoxifen promotes the formation of second-messenger lipids in cells. Integral in this biochemical response is the activation of cellular phospholipase D (PLD) by tamoxifen via a mechanism that appears to involve participation of PKC. We thus show that tamoxifen activates phospholipases and provokes translocation of PKC; in light of these findings, implications in the areas of cell signal transduction and proliferative control are far reaching. One distinct mechanism of cell signaling is via activation of phospholipases. The phospholipase hydrolysis products phosphatidic acid (PA) and diacylglycerol (DG) play a crucial role in regulating cell proliferation and differentiation (Asaoka *et al.,* 1992). Please note that in the naming of DG and the glycerophospholipids we do not distinguish here between the type of aliphatic linkage to glycerol (ester or ether). Therefore, from the standpoint of non-genomic actions, tamoxifen may participate in membrane responses that ultimately influence down-stream nuclear events. Because of widespread therapeutic use, ongoing prophylactic trials and the questions regarding endometrial insult (Andersson *et al.,* 1992), we have conducted this study to further our understanding of the molecular actions of tamoxifen.

MATERIAL AND METHODS

Cells

The CCD986SK cell line (human breast fibroblasts) was obtained from the ATCC (Rockville, MD). The cells are of finite lifespan and were used at passages 6–12. Cells were grown in DMEM, F-12, 50/50 mix (Mediatech, Herndon, VA) containing 10% FBS (Hyclone, Logan, UT), L-glutamine (584 mg/L), penicillin (100 units/ml) and streptomycin (100 µg/ml) GIBCO BRL (Grand Island, NY). Cells were passaged using 0.05% trypsin, 0.53 mM EDTA. Stock cultures were maintained in 75 cm^2 tissue culture flasks and subcultured into 6-well plates for experiments. Cultureware was from Corning-Costar (Cambridge, MA). Phenol red-free DMEM medium was purchased from GIBCO BRL, and charcoal dextran-treated FBS was from Hyclone.

Chemicals

Tamoxifen (free base), clomiphene, β-estradiol, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), phorbol dibutyrate (PDBu), Dowex-

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This paper is dedicated to the memory of Dr. Ann Sesko, our dear colleague and friend.

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50W, 50X4-200, ionic form hydrogen and fatty acid-free BSA were from Sigma (St. Louis, MO). 4-*Hydroxy* tamoxifen was a gift from D. Salin-Drouin (Paris, France). GF 109203X was purchased from BIOMOL (Plymouth Meeting, PA). Anti-PKC ϵ, affinity purified, was from GIBCO BRL. Polyacrylamide gels (4–20%) and Tris-glycine were purchased from Novex (San Diego, CA). Anti-rabbit IgG conjugated to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system were from Amersham (Arlington Heights, IL). [9,10-^{3}H(N)]Myristic acid (16–30 Ci/mmol), [2-^{3}H]glycerol (18.2 Ci/mmol) and [methyl-^{3}H]choline (80 Ci/mmol) were from DuPont NEN (Boston, MA). [1-^{3}H]Ethanolamine (30 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). EcoLume liquid scintillation fluid was from ICN (Costa Mesa, CA). Silica Gel G thin-layer chromatography (TLC) plates were purchased from Analtech (Newark, DE). Reagent grade solvents for TLC were from Fisher (Pittsburgh, PA). Phospholipids were from Avanti Polar Lipids (Alabaster, AL), and neutral lipids were from Nu Chek Prep (Elysian, MN).

Cell radiolabeling, treatment and lipid analysis

Cells, in 6-well plates, were labeled 1 day before confluence with [^{3}H]myristic acid (1.0 μCi/ml medium) for 24 hr. Cells were labeled for 3 days with [^{3}H]glycerol (10 μCi/ml phenol red-free medium, 5% charcoal-treated FBS). For all experiments employing [^{3}H]myristic acid, after radiolabeling, cell monolayers were rinsed 2 times with and equilibrated for 5 hr in phenol red-free medium containing 0.3 mg BSA/ml. After one final wash, cells were exposed to tamoxifen or other drugs as indicated. The following vehicles were used to dissolve and deliver the drugs to the culture medium: tamoxifen, 4-OH tamoxifen and estradiol (acetone); clomiphene (acetone/ethanol, 1:1, v/v). Control media contained vehicle. After cell treatment, media were aspirated, and total cellular lipids were extracted by the method of Bligh and Dyer (1959) modified to contain 2% acetic acid in the methanol.

[^{3}H]PA, [^{3}H]DG and [^{3}H]phosphatidylethanol (PEt) were resolved from total cellular lipids by TLC employing solvent systems previously described (Huang and Cabot, 1990; Cabot *et al.,* 1995). The lipids, identified by co-migration with commercial standards, were visualized in iodine vapor. Radioactivity was determined in the silica gel scrapings (after iodine subliming) by liquid scintillation spectrometry. Areas of the gel were first scraped into 0.5 ml water, 4 ml of EcoLume was added, and the contents of the plastic minivials were mixed before counting.

Water-soluble phospholipid metabolites were separated into base and phosphoryl-base moieties on a Dowex 50W acidic cation exchanger (1.0 ml bed volume) using Bio-Rad (Hercules, CA) Poly-prep 10 ml columns. Phosphobases were eluted with water (5–10 ml), and free bases were eluted with 10 ml 1.0 N HCl. Samples were assayed for radioactivity by liquid scintillation spectrometry.

Western-blot analysis of PKC

Cells were serum starved for 2 hr prior to the addition of tamoxifen or vehicle (acetone). After treatment, cells were scraped in cold lysis buffer, and cytosolic and membrane fractions were prepared according to the methods of Ha and Exton (1993). Protein was determined using the Pierce BCA Protein Assay Reagent system (Rockford, IL). Equal amounts of protein were loaded on a 4–20% SDS-PAGE. Separated proteins were transferred to nitrocellulose paper and analyzed for the presence of PKC ϵ using anti-PKC ϵ antibody. Bound antibody was visualized by ECL according to the manufacturer's protocol (Amersham).

All experiments were repeated at least 3 times, and all data points represent the mean ± SD from 3 separate cultures. Variation was less than 10% in figures in which error bars are not included.

RESULTS

The hydrolysis of phospholipids and the accompanying elevation of DG and PA are associated with signal events that govern cellular growth. The hydrolysis of phosphatidylcholine (PC), as opposed to polyphosphoinositides, is necessary for eliciting long-term responses such as control of proliferation and differentiation (Asaoka *et al.,* 1992). To study PC metabolism specifically we conducted experiments to determine whether myristic acid, which is preferentially incorporated into PC in many cell types (Huang and Cabot, 1990), would be useful to radiolabel PC of CCD986SK cells. We found that approx. 50% of the added [^{3}H]myristic acid was taken up by the cells after 24 hr. Of this, 80% of the tritium was localized in PC, 4.0% in phosphatidylethanolamine (PE), 3.5% in inositol- and serine-containing phospholipids, 10.5% in sphingomyelin and 0.5% in lyso-PC. In preliminary experiments using cells prelabeled with [^{3}H]myristic acid, we found that tamoxifen treatment caused a 2- to 3-fold increase in levels of cellular [^{3}H]PA and [^{3}H]DG within 30 min (data not shown). The kinetics and pharmacology of this response were subsequently investigated.

The influence of tamoxifen on the generation of cellular lipid second messengers was shown to be time and concentration dependent. The amount of radiolabeled PA and DG increased steadily with exposure time (Fig. 1*a*). Increases in cellular lipid second messengers were evident as early as 5 min after the introduction of tamoxifen. By 40 min, second messenger levels were increased to 2.8- and 2.4-fold above control for PA and DG, respectively. Over a concentration range of 2.5–20 μM, tamoxifen exposure caused steady increases in cell-associated PA and DG (Fig. 1*b*). At the maximum concentration tested (20 μM), tamoxifen induced a 2.5- and 2.0-fold increase in cellular PA and DG, respectively. Examination of tamoxifen-induced PA and DG formation over extended times (60–120 min) demonstrated that whereas PA peaked at 60 min and slowly decreased thereafter, DG levels peaked at 60 min and were sustained throughout the 120 min time course.

When cells are challenged with agonists, the principal enzymatic pathways for the formation of PA are through the action of either PLD or DG-kinase. To determine whether tamoxifen activates cellular PLD, we conducted experiments with ethanol in the medium. This enables formation of PEt, the transphosphatidylation product of PLD, at the expense of PA. Figure 2 reveals that tamoxifen activates cellular PLD, as evidenced by the generation of PEt. Activation of PLD was dose dependent with respect to tamoxifen concentration. Cellular PEt increased most markedly between 10 and 20 μM tamoxifen, a concentration range that also elicited the greatest production of cellular DG and PA (Fig. 2). PA production was biphasic, with a slight increase from 0 to 10 μM and a marked increase between 10 and 15 μM. At the highest concentration evaluated (20 μM), tamoxifen elicited a 5-, 3.2- and 2.3-fold increases in PEt, PA and DG, respectively. The lipid responses elicited by tamoxifen are reminiscent of the rapid signal responses produced by hormones and growth factors acting via phospholipase-coupled pathways (Huang and Cabot, 1990).

The influence of lower dose and increased exposure time was evaluated over a 2–4-day time course. In these experiments cells were cultured continually in the presence of tamoxifen and [^{3}H]glycerol. Preliminary results revealed that the levels of cellular DG were increased by 40% (control = 953 ± 4.7 cpm; tamoxifen-treated = 1,344 ± 118 cpm) after 4 days with 2.5 μM tamoxifen (data not shown). The data of Figure 3 show the influence of low-dose tamoxifen on the level of cellular DG over a 3-day exposure period. Tamoxifen promoted a dose-dependent increase in cellular [^{3}H]DG.

The data of Figure 2 suggest that other phospholipases, in addition to PLD, are activated by tamoxifen. To investigate this in more depth at the biochemical level, we conducted an experiment using increased amounts of ethanol. The data of Figure 4 reveal that: 1) the level of tamoxifen-induced DG is unaffected by increasing the amount of ethanol in the medium; 2) PEt increases proportionally with respect to increasing ethanol; and 3) PA decreases (at the expense of PEt) with increasing ethanol. Overall, these data indicate that tamoxifen activates PLD and phospholipase

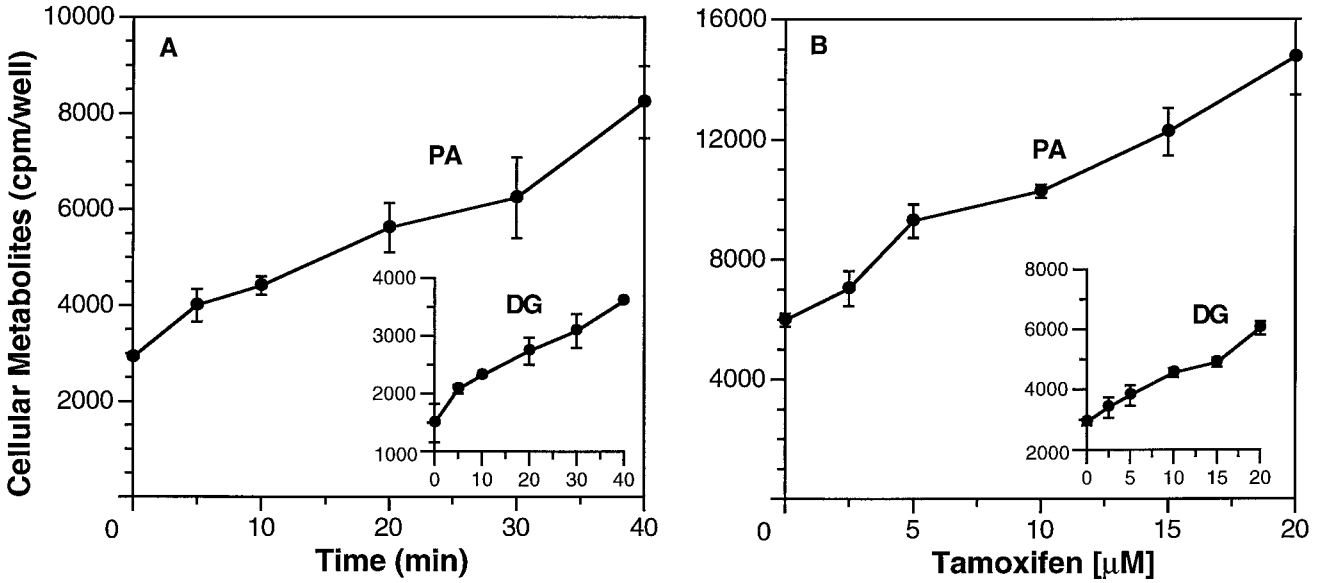

FIGURE 1. – Influence of tamoxifen on the generation of PA and DG in CCD986SK cells. Cells were prelabeled with [^{3}H]myristic acid and equilibrated for 5 hr prior to addition of 10 μM tamoxifen *(a)* or tamoxifen concentrations indicated in *b* (20 min). Cellular lipids were analyzed by TLC as detailed in the methods section.

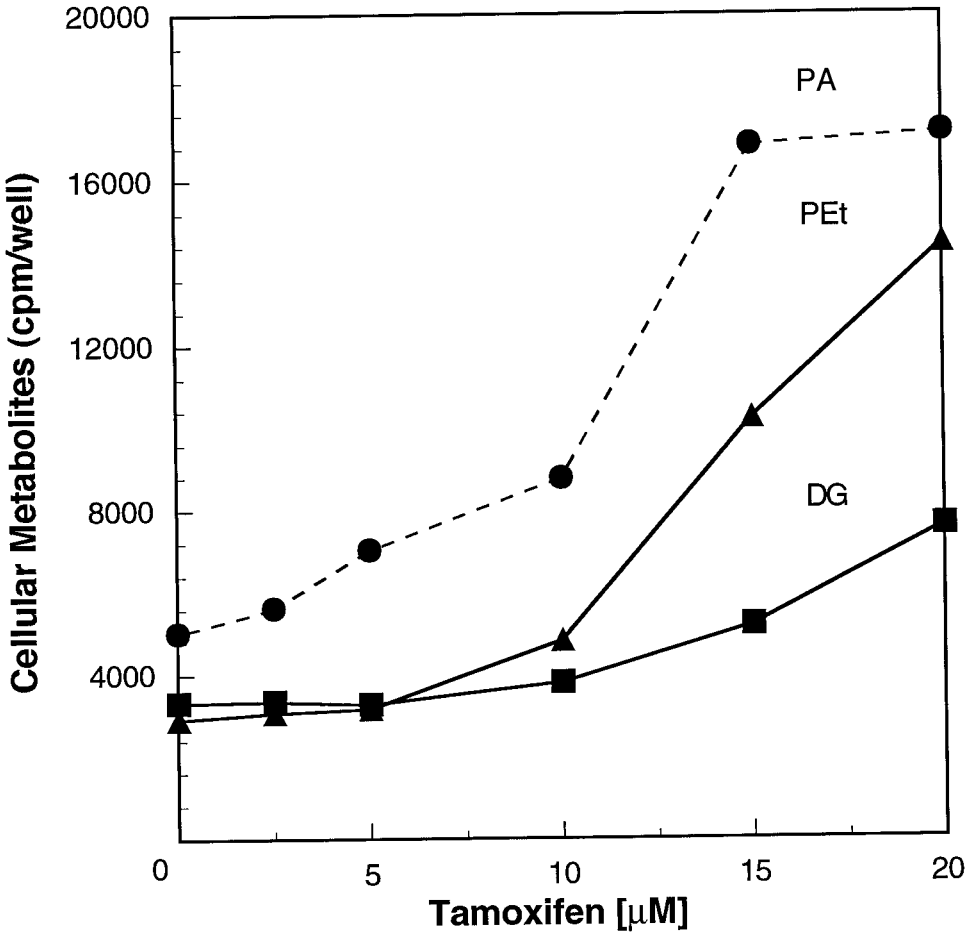

FIGURE 2. – Tamoxifen treatment elicits PLD activity as evidenced by PEt generation in CCD986SK cells. [^{3}H]myristate-labeled cells were equilibrated for 5 hr before addition of tamoxifen, at the indicated concentrations, in medium containing 1% ethanol. Cells were treated for 30 min. PLD activity was measured by the formation of PEt.

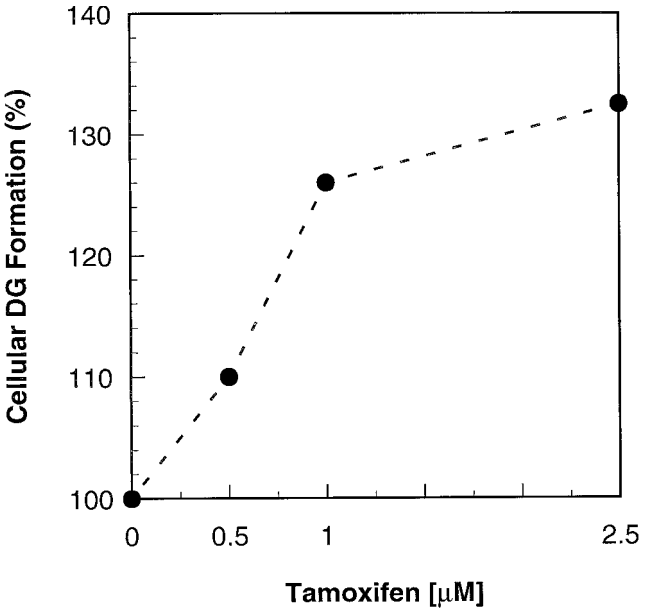

FIGURE 3. – The influence of continuous tamoxifen exposure on [^{3}H]DG metabolism in CCD986SK cells. Cells were cultured with [^{3}H]glycerol (during log phase growth) for 3 days in phenol red-free DMEM containing 5% charcoal/dextran-treated FBS and tamoxifen at the indicated concentrations. After 3 days, the medium was removed, cultures were rinsed with ice-cold PBS and cellular lipids were extracted and analyzed. Data points are from the average of triplicate cultures. Error bars are not shown; average ± SD ≤ 5.0%.

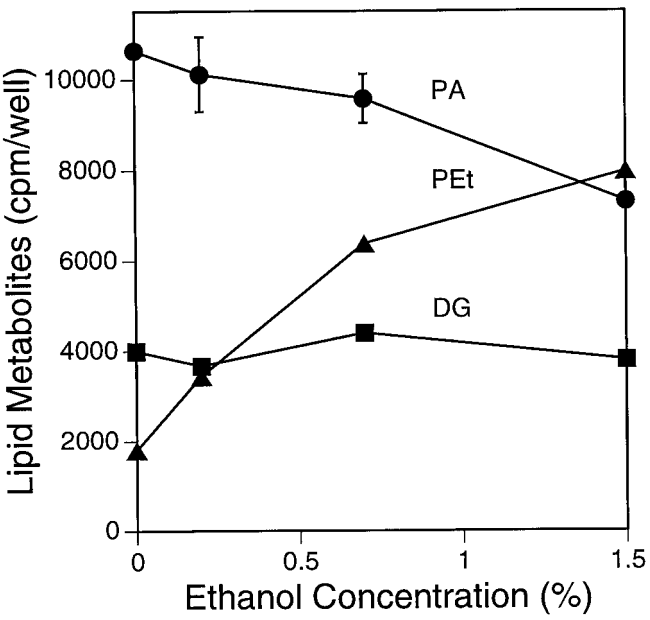

FIGURE 4. – The influence of increasing ethanol in culture medium on tamoxifen-activated formation of PEt, DG and PA. Cells were prelabeled with [^{3}H]myristic acid and equilibrated as described. Treatment media (15 μM tamoxifen) were made to contain 0–1.5% ethanol, as indicated, and cells were treated for 20 min. Lipid metabolites were resolved by TLC. DG, in the absence of tamoxifen, was 1,739 cpm/well. DG in the presence of tamoxifen with 0% ethanol was 4,000 cpm, as indicated on the y-axis. Data points represent triplicate cultures; the absence of error bars indicate error ≤5%.

C (PLC), the latter demonstrated by continuous formation of DG, free from the influence of ethanol.

To investigate tamoxifen-induced PLC activation further and disclose the cellular lipid sources of PA and DG, cells were labeled with [^{3}H]choline and [^{3}H]ethanolamine and treated with tamoxifen. The data of Table I establish that tamoxifen activates hydrolysis of both cellular PC and PE and that phospholipases C and D are stimulated. Tamoxifen elicited a 6.3- and 4.4-fold increase in release to the medium of choline- and ethanolamine-containing water-soluble metabolites. Examination of these metabolites showed that they were comprised of both choline and phosphorylcholine and ethanolamine and phosphorylethanolamine. Tamoxifen-stimulated PC hydrolysis by PLD is indicated by the 3.1-fold increase (3,373 *vs.* 10,608 cpm) in choline production, while PC hydrolysis by PLC is shown by the 10-fold increase (2,873 *vs.* 28,681 cpm) in phosphorylcholine production. Similarly, tamoxifen stimulated the hydrolysis of PE by PLD (3-fold increase in ethanolamine) and by PLC (5.8-fold increase in phosphorylethanolamine). PE hydrolysis was also evidenced by examining cytoplasmic metabolites (last category, Table I), in which tamoxifen caused increases in both ethanolamine and phosphorylethanolamine.

In further support of the hypothesis that tamoxifen promotes degradation of PC and PE, we examined the levels of these lipids in cells after treatment with tamoxifen. Cells were labeled with [^{3}H]myristic acid and treated with tamoxifen (20 μM) for 20 min in medium containing 1% ethanol. A 6.2-fold increase in cellular PEt was accompanied by losses of PC and PE radioactivity of 7.2 and 13.8%, respectively. Therefore, in cells treated with tamoxifen, increases in PEt are accompanied by decreases in PC and PE.

The structural requirements of tamoxifen for PLD activation were evaluated using a series of analogous compounds and the tumor promoter TPA. The data of Figure 5 demonstrate that tamoxifen was the most potent activator of cellular PLD (Fig. 5*a*), whereas 4-OH-tamoxifen was the least active. Tamoxifen produced a >7-fold increase in cellular PEt, and 4-*hydroxy*-tamoxifen elicited a 0.35-fold increase. TPA, a known activator of PLD

TABLE I – INFLUENCE OF TAMOXIFEN ON PC AND PE METABOLISM IN CCD986SK CELLS[1]

Sample	Phospholipid metabolism[2]	
	Control	Tamoxifen
[^{3}H]choline-labeled cells		
Total counts released into medium	6,246	39,289
Choline	3,373	10,608
Phosphorylcholine	2,873	28,681
[^{3}H]ethanolamine-labeled cells		
Total counts released into medium	23,174	101,363
Ethanolamine	11,378	33,044
Phosphorylethanolamine	11,795	68,318
Total counts in cytoplasm	70,136	121,410
Ethanolamine	5,611	10,198
Phosphorylethanolamine	64,525	111,211

[1]Cultures were labeled with [^{3}H]choline (7.0 μCi/ml) and [^{3}H]ethanolamine (3.8 μCi/ml) for 48 hr in medium containing 5% FBS. After 48 hr, uptake was 11 and 50% for choline and ethanolamine, respectively. Following equilibration and rinse, cells were treated with control medium or tamoxifen-containing medium (20 μM) for 30 min at 37°C. For [^{3}H]choline-labeled cultures, treatment medium contained 15 mM unlabeled choline supplement, and in [^{3}H]ethanolamine cultures, treatment medium contained 2.5 mM unlabeled ethanolamine supplement. After 30 min, media were analyzed directly (total counts released to medium) by counting an aliquot in scintillation fluid. Resolution of base and phosphoryl-base groups in medium was done by column chromatography (see Material and Methods). Cytoplasmic metabolites were isolated in the upper aqueous-methanolic phase of the cellular lipid extract.–[2]Denotes total cpm/culture released to the medium (underlined) or in cytoplasm (underlined) and the constituent cpm in respective base and phosphoryl-base groups.

(Cabot *et al.*, 1989), also stimulated PLD activity as shown by a 3.5-fold increase over control in PEt anabolism. The structure-activity relationship for activation of PLD, in descending order, was as follows: tamoxifen > clomiphene > TPA > estrogen >

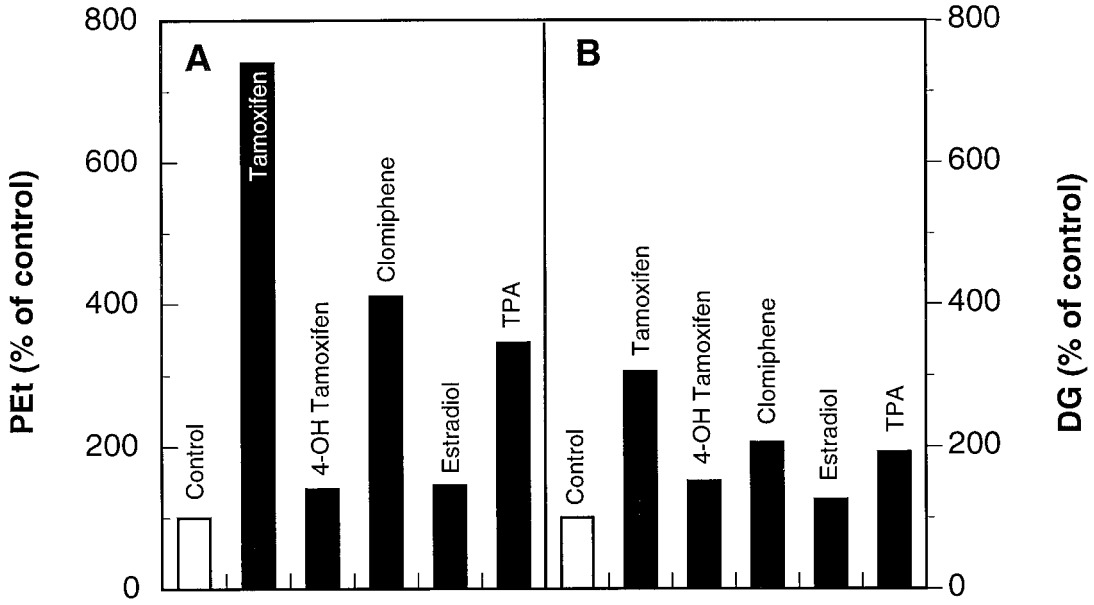

FIGURE 5. – The influence of various triphenylethylenes, estrogen and TPA on PLD activity in cultured CCD986SK cells. Cultures were radiolabeled with [^{3}H]myristic acid, equilibrated and treated with the compounds for 30 min in medium containing 1% ethanol. All agents were tested at 15 μM except for TPA (50 nM). PEt and DG were resolved from total cellular lipids by TLC.

4-OH tamoxifen. A similar structure-activity relationship for stimulated production of cellular DG was noted (Fig. 5*b*). Tamoxifen and clomiphene treatment produced 3- and 2-fold increases, respectively, in cellular DG. During the course of this work we have noted the degree of PLD activation by tamoxifen to be variable. Activation ranges between 2.5- and 7-fold. We have not ascertained whether this is due to passage number or degree of confluency of the monolayer. There is some indication that activation is higher at lower cell passage.

We examined the effects of PKC down-regulation and the influence of GF 109203X, a PKC inhibitor, on tamoxifen-induced PLD activity. The data of Figure 6 demonstrate that down-regulation of PKC by overnight exposure of cells to PDBu totally abolished TPA-induced PLD activity and blocked tamoxifen-induced PLD activation by 80%. Similarly, GF 109203X at 2.5 and 5.0 μM diminished tamoxifen stimulation of PLD activation by 46 and 61%, respectively. Thus, it is suggested that tamoxifen activates PLD in a PKC-dependent manner.

A major pathway of PLD activation by extracellular signals involves PKC translocation and activation (Asaoka *et al.,* 1992). To find out whether PLD activation by tamoxifen is mediated by PKC, we examined the effect of tamoxifen on PKC movement in CCD986SK cells. The data of Figure 7 show that PKC ϵ is equally distributed within the cytosol and membranes of the cells. Addition of tamoxifen causes depletion of PKC ϵ from the cytosol and concomitant translocation of the enzyme to the membrane. Other isoforms tested (α, β, γ, ζ) were not responsive to tamoxifen treatment (data not shown). As we propose that tamoxifen provokes PLD through a PKC-governed mechanism, it was of interest to conduct a more precise time course of PKC translocation. In cell cultures treated with 15 μM tamoxifen, depletion of PKC from cytosol was evidenced as early as 5 min.

We have begun to assess the ability of tamoxifen to induce second-messenger formation in different types of cells. In MDA-MB-231 cells (estrogen receptor-negative), tamoxifen elicits PA generation (Cabot *et al.,* 1995). Preliminary experiments with MCF10A cells, a human mammary epithelial cell line often used as a ''normal'' control, indicate that tamoxifen exposure promotes an increase in the levels of cell-associated DG (data not shown).

DISCUSSION

The experiments here provide insight into estrogen receptor-independent interactions of tamoxifen and thereby shed light on possible non-genomic mechanisms of action of this therapeutic agent. Tamoxifen is widely used for treatment and prevention of breast cancer. This popularity comes not without controversy. The incidence of uterine cancer increased in breast cancer patients receiving tamoxifen (Andersson *et al.,* 1992). This suggests that tamoxifen may have long-term effects on other areas of the body even as it inhibits the growth of breast cancer cells. The benefits, however, appear to outweigh the risk.

The broad utility of tamoxifen for treatment of breast cancer, neoplasms of brain (Couldwell *et al.,* 1993) and melanoma (Del Prete *et al.,* 1984) is alone suggestive of multimodal mechanisms of action, some of which are independent of the estrogen receptor (Kellen, 1996). Reports have surfaced demonstrating that not all tamoxifen-related biological effects are mediated via estrogen receptor interactions. Tamoxifen has been shown to induce transforming growth factor-β production in human breast stromal components (Butta *et al.,* 1992), while estrogen was ineffective. Also of importance to issues of pharmacological activity are studies demonstrating multiple binding sites for anti-estrogens (Brandes *et al.,* 1987; Gross *et al.,* 1993). In addition, anti-estrogens have been shown to modify the activity of growth factors important for cellular proliferation; however, a direct link with signal transduction has not been provided.

Our work demonstrates an association among tamoxifen, PKC ϵ translocation and phospholipase activation. Phospholipases are essential for membrane maintenance and crucial for cellular hormone and growth factor signaling. We have shown that tamoxifen activates PLD, resulting in PA formation. In addition, the data show that cellular DG increases in response to tamoxifen exposure, and in the presence of increasing ethanol, DG is produced in an unabated fashion (Fig. 4). By following metabolism of the hydrophilic portion of phospholipids (Table I), we have ascertained that DG can arise independently by the action of PLC. Our experiments have also revealed that the cellular source of PA and DG is both PC and PE. Because experiments were conducted using radiotracers, we do not have quantitative information on the contribution of PC and PE to second-messenger formation. The influence of tamoxifen on formation of DG, a physiological

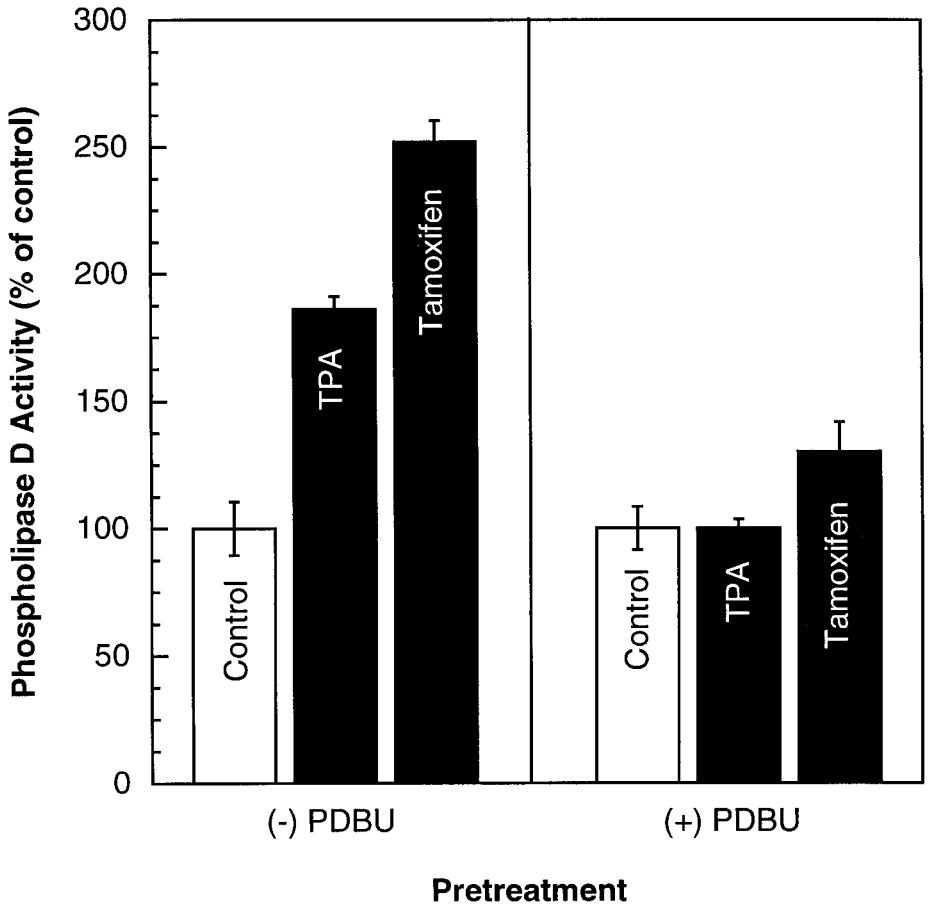

FIGURE 6. – Effect of PKC down-regulation on TPA- and tamoxifen-stimulated PLD activity. Cells were cultured with [^{3}H]myristic acid ± PDBu (1.0 μM) for 18 hr. After a 5 hr equilibration in serum-free, isotope-free medium (±PDBu), cells were challenged with TPA (50 nM) or tamoxifen (20 μM) for 20 min. PLD activity was measured by the formation of PEt. The presence of PDBu (18 hr) did not alter cellular incorporation of myristic acid.

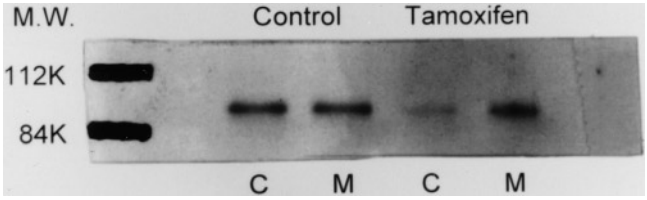

FIGURE 7. – The influence of tamoxifen on PKC ϵ translocation in CCD986SK cells. Cells were grown to confluence (10 cm dishes) and briefly serum starved (3 hr) in phenol red-free DMEM containing BSA (0.3 mg/ml). Cultures were then treated in the absence (acetone vehicle) or presence of tamoxifen (20 μM) for 20 min. Isolation of cytosol and membranes, SDS-PAGE, transblotting and immunoblotting with PKC isoform-specific antibodies and ECL (Amersham) detection are as described in Material and Methods. For SDS-PAGE, 7.5 μg of cell protein were added per sample lane. The addition of isozyme-specific PKC ϵ peptide blocked the anti-PKC ϵ reaction. MW, molecular weight markers; C, cytosol; M, membrane.

activator of PKC (Asaoka *et al.,* 1992), and PA, another important lipid signaling molecule (reviewed in Cabot *et al.,* 1995), has wide-ranging implications. In the human breast cancer cell line MDA-MB-231, we have shown that tamoxifen stimulates PA formation by a pathway that likely recruits DG-kinase (Cabot *et al.,* 1995).

Hydrolysis of PC with formation of PA and DG is associated with sustained activation of PKC. Such sustained activity is necessary for eliciting long-term responses, *e.g.,* control of cellular proliferation and differentiation (Asaoka *et al.,* 1992). Modified second-messenger metabolism, elicited by tamoxifen, may ultimately lead to changes in cell growth and in the impact of hormones and growth factors on target tissues. Additionally, the ability of tamoxifen to reverse multidrug resistance may be partially associated with membrane lipid changes. In multidrug-resistant human cancer cells, tamoxifen markedly retards glycosphingolipid metabolism (Cabot *et al.,* 1996). Tamoxifen has also been shown to increase the rate of PC synthesis in multidrug-resistant leukemia (Ramu *et al.,* 1991).

The amount of tamoxifen required to induce phospholipase activation is in the low micromolar range. Although this is not in line with test tube pharmacokinetics for blocking the estrogen receptor, patients receiving 10 mg tamoxifen twice a day are not limited to attaining pharmacokinetic levels in circulation. In patients receiving tamoxifen, serum concentrations are around 0.1–0.5 μM, with intratumoral levels 5 to 10 times higher (Lien *et al.,* 1991; Johnson *et al.,* 1993). In Figure 3 we showed that tamoxifen (1.0 μM), over extended time, increased the level of cell-associated DG. These experiments raise important questions regarding DG levels in tamoxifen-responsive tissues of the body. Additionally, the new direction for use of high-dose tamoxifen in treatment of cancer (Smith and Trump, 1995) is not founded on physiological and/or pharmacokinetic relationships with the estrogen receptor. In this instance the significance of tamoxifen transmembrane signaling and interaction with cell surface proteins may

be related to the multi-model influence of this drug. It could be a matter of concern that the higher concentrations of tamoxifen (up to 20 μM) used in this study may cause cell lysis and non-specific changes in membranes. We did not observe toxicity over the short time periods examined. Furthermore, the data of Figure 3, derived using lower dose tamoxifen (0.5–2.5 μM) for 3 days of treatment, illustrate a condition of a more physiological nature, in which tamoxifen was employed at an amount to reflect serum and intratumoral levels. This 3-day treatment was likewise non-toxic.

The mechanism by which tamoxifen activates PLD and increases cellular PA and DG appears to be through a PKC-directed pathway. In an estrogen receptor-deficient MCF-7 subline, tamoxifen has been shown to stimulate PLD (Kiss, 1994). Our work demonstrates a relationship among tamoxifen exposure, translocation of PKC and phospholipase activation. This corroborates our earlier finding, which showed that PLD is activated by a PKC-dependent avenue (Cabot *et al.*, 1989). The data herein show that phorbol esters likewise stimulate PLD activity in CCD986SK cells. Both TPA- and tamoxifen-induced PLD activity were diminished by elements known to block PKC, namely, down-regulation and the inhibitor, GF 109203X. In addition to these indirect experiments, we provide evidence showing that tamoxifen elicits translocation of PKC ϵ from cell cytosol to membrane. Translocation to membrane was noted as early as 5 min after addition of tamoxifen. It is generally accepted that such translocation of PKC results in its activation (Asaoka *et al.*, 1992). Interestingly, PKC ϵ was the only isoform found to be responsive to tamoxifen. In renal mesangial cells, activation of PLD by extracellular adenine nucleotides is mediated by PKC ϵ (Pfeilschifter and Merriweather, 1993), and overexpression of PKC ϵ enhances phospholipase C activity in NIH 3T3 cells (Kiss and Garamszegi, 1993). Our data strongly suggest that tamoxifen elicits phospholipase activation by way of PKC, although there are mixed reports regarding anti-estrogen influence on PKC activity (O'Brian *et al.*, 1985; Bignon *et al.*, 1991). *In vitro* experiments conducted with partially purified rat brain PKC, revealing 50% inhibition of PKC with 100 μM tamoxifen (O'Brian *et al.*, 1985), may not be easily compared with our experiments with intact cells. Differential effects of tamoxifen on PKC are observed in intact cells *vs.* disrupted cells (Issandou *et al.*, 1990). PKC activation by triphenylethylenes has been observed in intact cells (Bignon *et al.*, 1991), in which, from structure-activity relationship studies, it was suggested that the basic amino side-chain interacts with the regulatory domain and the 1,1*bis* (p-hydroxyphenyl) ethylene moiety interacts with the catalytic domain of PKC. Additionally, earlier studies on tamoxifen and PKC were conducted before much was known about the PKC isoforms, some of which display strict co-factor requirements and have specific physiological roles (Asaoka *et al.*, 1992; Nishizuka, 1995). We have also shown that tamoxifen causes selective translocation of PKC ϵ in breast cancer MCF-7 cells (Lavie *et al.*, 1996). Further insight into the estrogen receptor-independent activities of tamoxifen should be beneficial for the design of new therapeutic agents.

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