Int. J. Cancer: **77,** 928–932 (1998)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

TAMOXIFEN INDUCES SELECTIVE MEMBRANE ASSOCIATION OF PROTEIN KINASE C EPSILON IN MCF-7 HUMAN BREAST CANCER CELLS

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Tamoxifen, a synthetic antiestrogen, is known for its antitumoral action *in vivo;* however, it is well accepted that many tamoxifen effects are elicited via estrogen receptor-independent routes. Previously, we reported that tamoxifen induces PKC translocation in fibroblasts. In the present study, we investigated the influence of tamoxifen, and several triphenylethylene derivatives, on protein kinase C (PKC) in MCF-7 human breast cancer cells. As measured by Western blot analysis, tamoxifen elicited isozyme-specific membrane association of PKC-ϵ, which was time-dependent (as early as 5 min post-treatment) and dose-dependent (5.0–20 μM). Tamoxifen did not influence translocation of α, β, γ, δ or ζ PKC isoforms. Structure-activity relationship studies demonstrated chemical requirements for PKC-ϵ translocation, with tamoxifen, 3-OH-tamoxifen and clomiphene being active. Compounds without the basic amino side chain, such as triphenylethylene, or minus a phenyl group, such as N,N-dimethyl-2-[(4-phenylmethyl)phenoxy]ethanamine, were not active. *In vitro* cell growth assays showed a correlation between agent-induced PKC-ϵ translocation and inhibition of cell growth. Exposure of cells to clomiphene resulted in apoptosis. Since PKC-ϵ has been associated with cell differentiation and cellular growth-related processes, the antiproliferative influence of tamoxifen on MCF-7 cells may be related to the interaction with PKC-ϵ. *Int. J. Cancer* 77:928–932, 1998.
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Tamoxifen is widely used in the treatment of breast cancer, and it is currently in trials for breast cancer prevention. This synthetic antiestrogen is also used in the treatment of melanoma (Del Prete *et al.,* 1984) and malignant glioma (Couldwell *et al.,* 1993). Apart from blocking the growth-promoting influence of estrogen on mammary cancer cells, many of the cellular responses elicited by tamoxifen are independent of the estrogen receptor (ER) (Kellen, 1996), and patients with ER^- breast cancer, as well as ER^- breast cancer cell lines, respond to tamoxifen. Studies have shown that tamoxifen provokes multiple cellular responses, including induction of transforming growth factor-β_1 production (Butta *et al.,* 1992), calmodulin antagonism (Hardcastle *et al.,* 1995), modification of lipid second-messenger formation (Cabot *et al.,* 1997; Croxtall *et al.,* 1997), interaction with and reversal of P-glycoprotein (P-gp)-mediated multidrug resistance (Kirk *et al.,* 1994) and inhibition of glycosphingolipid synthesis (Cabot *et al.,* 1996). Tamoxifen is thought to interact with protein kinase C (PKC), a Ca^{2+}- and phospholipid-dependent enzyme that plays a key role in transmembrane signaling of a wide variety of stimuli including growth factors (Magnuson *et al.,* 1994). Molecular cloning studies have shown that PKC is a family of closely related isozymes which comprise at least 11 different members including the calcium-dependent (α, β, γ), calcium-independent (δ, ϵ, η, θ, μ) and atypical (ζ, λ) isozymes. Most isozymes of PKC are activated by diacylglycerol and phorbol diesters, with PKC-ζ being the exception (Magnuson *et al.,* 1994). Upon stimulation, the enzyme undergoes translocation from the cytosol to the plasma membrane, a hallmark demonstrating activation.

PKC isozymes are differentially expressed in various cells. For example, MCF-7 wild-type cells express mainly PKC-β, -δ and -ϵ, with minor PKC-α content, whereas MCF-7 doxorubicin-resistant cells show a large increase in PKC-α and large decrease in PKC-δ and -ϵ levels (Blobe *et al.,* 1993). PKC isozymes may participate in different cellular processes. For example, PKC-ϵ may specifically mediate the cellular differentiation process (Hundle *et al.,* 1995; Ponzoni *et al.,* 1993), and PKC-ϵ appears to be responsible for regulation of cell adhesion to the extracellular matrix (Chun *et al.,* 1996). In addition, PKC-ϵ has been shown to mediate activation of phospholipase D (Pfeilschifter and Merriweather, 1993). In this study, we demonstrate that tamoxifen and related triphenylethylene analogs induce specific membrane translocation of PKC-ϵ in human breast cancer MCF-7 cells, and compare the relationship of PKC-ϵ translocation by triphenylethylenes to growth status of MCF-7 cells.

MATERIAL AND METHODS

Cells

MCF-7 human breast cancer cells were kindly provided by Dr. K.H. Cowan and Dr. M.E. Goldsmith, National Cancer Institute, Bethesda, MD. Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS; v/v), 100 units/ml of penicillin, 100 μg/ml of streptomycin and 584 mg/l of L-glutamine. Cells were grown in a humidified 6.5% CO_2 atmosphere tissue culture incubator and subcultured once a week using 0.05% trypsin and 0.53 mM EDTA solution.

Material

Leupeptin, aprotinin, dithiothreitol (DTT) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma (St. Louis, MO). Anti-PKC-α, -β, -λ -γ, -δ, -ζ and -ϵ antibodies (with

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The views expressed herein are those of the authors and do not necessarily reflect the views of the U.S. Army, U.S. Navy, Uniformed Services University of the Health Sciences or the Department of Defense.

Presented at the Annual Meeting of the American Association of Cancer Research, Washington, DC, 1996.

Grant sponsor: Breast Cancer Research Program of the University of California; Grant number: 0211; Grant sponsors: Ben B. and Joyce E. Eisenberg Foundation, Fashion Footwear Association of New York (FFANY) Shoes on Sale® and Cancer Research Campaign, United Kingdom.

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Received 4 February 1998; Revised 30 March 1998

respective competing peptides) and RPMI-1640 cell culture medium were purchased from GIBCO BRL (Gaithersburg, MD). Gradient 4–20% precast sodium dodecyl sulfate (SDS)-polyacrylamide gels were purchased from Novex (San Diego, CA). Bio-blot nitrocellulose blotting membranes were from Costar (Cambridge, MA). Bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL). FBS was from HyClone (Logan, UT) and cultureware was from Corning-Costar (Cambridge, MA). Tamoxifen analogs were prepared by Dr. McCague at the Institute for Cancer Research as follows: N,N-dimethyl-2-[(4-phenylmethyl) phenoxy]ethanamine (Rowlands *et al.*, 1990), the diethyl analog of tamoxifen (Jarman and McCague, 1985), and triphenylbut-1-ene (Schneider *et al.*, 1982). 3-OH-tamoxifen (droloxifene) was a gift from Pfizer Central Research (Groton, CT) and 4-OH-tamoxifen was a gift from Dr. D. Salin-Drouin (Besins Iscovesco, Paris, France).

PKC translocation

Cells were grown to near confluency in 15 cm tissue culture dishes. The cell monolayers were washed twice with serum-free RPMI-1640 medium containing 0.1% bovine serum albumin (BSA) and preincubated in the same for 2 hr at 37°C. Thereafter, either tamoxifen, tamoxifen analogs or TPA was added for indicated times. Cells were rinsed with ice-cold phosphate buffered saline (PBS; pH 7.4) and scraped in 1.0 ml of buffer A, containing 25 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 0.2 mg/ml Triton X-100. The cells were homogenized (over ice) 30 times using a Dounce homogenizer with a tight-fitting pestle, and the lysate was centrifuged at 100,000*g* for 30 min at 4°C (Beckman TL-100, Palo Alto, CA). The supernatant (cytosolic fraction) was collected, and the membrane pellet was suspended in 1.0 ml of buffer A, containing 10 mg/ml Triton X-100. The membrane suspension was sonicated with a microtip for 5 sec (KONTES, Micro Ultrasonic Cell Disrupter, Vineland, NJ), rocked for 30 min at 4°C and then centrifuged at 100,000*g* for 30 min. The supernatant was designated as the membranous fraction. Protein was determined using BCA protein assay reagents.

Immunoblot analysis

Samples containing equal amounts of protein (20–50 µg) were loaded in each lane for gradient 4–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run using a Mini-protein II gel system (Bio-Rad, Hercules, CA). Following electrophoresis, proteins were transferred onto nitrocellulose membranes using a Mini-blot apparatus (Bio-Rad). The immunoblots were incubated with PBS (pH 7.4) containing 5% milk powder for 1 hr and then incubated overnight with diluted specific anti-PKC antibody (1.0–2.0 µg/ml). To verify the specificity of antibodies in any of these applications, competing peptide (1.0–2.0 µg/ml) of the respective PKC isozyme was included in parallel immunodetection analysis. Peptide was used at a 1:1 (w/w) ratio to antibody and was incubated with the antibody for 30 min prior to use. Bound PKC antibodies were visualized by an enhanced chemiluminescence (ECL) detection system, according to the manufacturer (Amersham, Arlington Heights, IL), using horseradish peroxidase conjugated to anti-rabbit IgG as the secondary antibody. To reprobe the membranes with a different antibody the membranes were submerged in stripping buffer [0.1 M 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7)] and incubated with agitation at 50°C for 30 min. Thereafter, the membranes were washed 3 times with PBS, blocked in PBS containing 5% milk powder and probed as described above.

Cytotoxicity assay

Perimeter wells in our 96-well assays are not used for cells but rather contain 0.2 ml water. Cells were seeded (2,000 cells/well) in 0.1 ml RPMI-1640 medium containing 5% FBS and incubated at 37°C for 24 hr before drug addition. Drug was added in medium (0.1 ml), and the cells were incubated at 37°C for an additional 4 days. Vehicle (acetone, ethanol) was always added to control (minus drug) wells. The cytotoxic activity of a drug was determined using the Promega (Madison, WI) CellTiter 96 AQ_{ueous} cell proliferation assay kit. Each experimental point was performed in 6 replicates. Promega solution (only 20 µl) was aliquoted into each well, and the cells were incubated for 2–3 hr, or until an optical density (O.D.) of approximately 1.0 was obtained as a highest reading. Absorbance at 490 nm was recorded using an ELISA plate reader (Molecular Devices, San Diego, CA).

Apoptosis assay

Control and clomiphene-treated cells were grown in 10 cm culture dishes for 72 hr. Cells were harvested by trypsin-EDTA, centrifuged and then incubated with digestion buffer (10 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl, 0.5% SDS, 0.3 mg/ml proteinase K, pH 8.0) at 45°C for 18 hr. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and precipitated in ½ vol 7.5 M ammonium acetate and 2 vol 100% ethanol at −20°C, overnight. The preparation was centrifuged for 20 min at 10,000*g*, 4°C. RNA contaminate was digested in buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, 0.1% SDS and 100 units/ml RNase, at

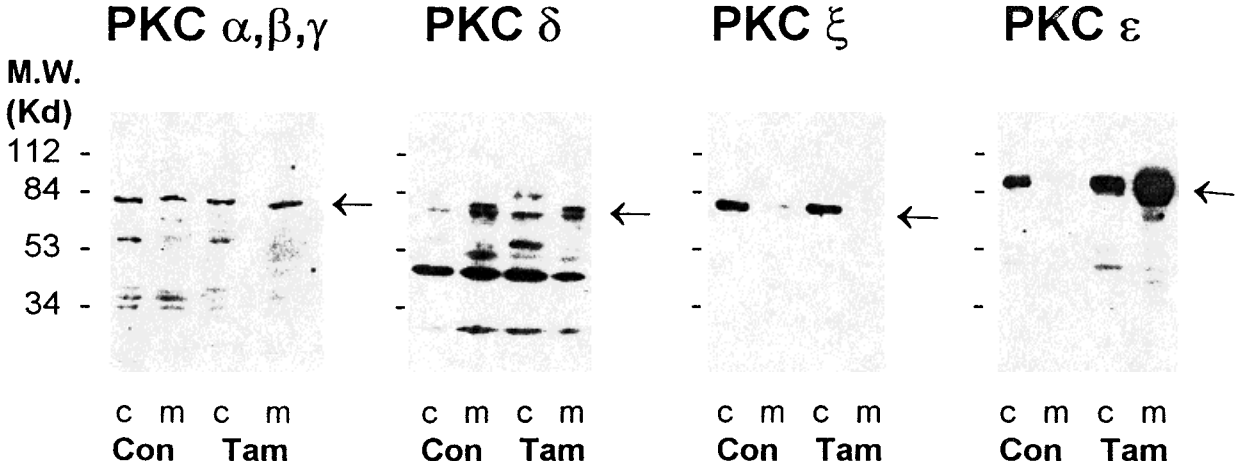

FIGURE 1 – Tamoxifen treatment induces isozyme-selective translocation of PKC-ε in MCF-7 cells. Cells were either untreated controls (Con) or treated with 20 µM tamoxifen (Tam) for 20 min. Thereafter, cell fractionation and immunoblot analysis, using different isozyme-specific anti-PKC antibodies, were performed as described in Material and Methods. Equal amounts of cytosolic (c) and membrane (m) protein were loaded per lane. Arrows indicate position of PKC isozymes.

37°C for 2 hr. Re-extracted DNA (6 μg) was analyzed by electrophoresis on a 2% agarose gel in TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.3).

RESULTS

Previous studies have indicated the presence of various PKC isozymes in MCF-7 cells using Western blot analysis (Blobe *et al.*, 1993). In this study, we have assessed PKC isozyme translocation in MCF-7 cells in response to the antiestrogen tamoxifen. As shown in Figure 1, tamoxifen caused preferential translocation of PKC-ϵ, but not -α, -β, -λ -γ, -δ or -ζ isozymes. The immunoblot demonstrates that whereas membrane fractions from control cells (minus tamoxifen) contain little PKC-ϵ protein, membranes from tamoxifen-treated cells show a marked elevation in PKC-ϵ levels.

The effective concentration of an antitumor drug is relevant to its clinical use. Figure 2 shows that tamoxifen, at 5.0 μM, induces PKC-ϵ translocation from cytosol with concomitant elevation in membranes. Clinical treatment with tamoxifen typically results in 0.1–0.5 μM drug concentrations in serum, but with intratumoral levels 5–10 times higher (Johnston *et al.*, 1993). To determine the time frame for tamoxifen-induced PKC-ϵ translocation, cells were treated with tamoxifen (1–60 min), and fractionated cytosol and membranes were subjected to immunoblot analysis. As shown in Figure 3, cytosolic PKC-ϵ levels began to decrease as early as 5 min and disappeared almost completely by 20 min.

Tamoxifen has distinct structural groupings comprised of a large hydrophobic domain (triphenyl) and a hydrophilic domain (chargeable amino group). We investigated various structural analogs of tamoxifen for their ability to translocate PKC-ϵ. First, as shown in Figure 4, TPA elicited marked translocation of PKC-ϵ from cytosolic component to membranes. This TPA-induced redistribution serves as an indicator of maximal translocation effect, in comparison to an equal distribution of PKC-ϵ between cytosol and membranes of untreated cells. Application of the triphenylethylene analogs 3-OH-tamoxifen, 4-OH-tamoxifen and clomiphene produced a major reduction in PKC-ϵ levels in MCF-7 cell cytosol with a concomitant increase in membranes. In contrast, ''right-side'' tamoxifen, which lacks 1 of the phenyl groups, was completely inactive. The same was true for triphenylethylene, which has all phenyl constituents but is devoid of the side chain. Lastly, diethyltamoxifen, which contains 2 ethyl groups on the amino dimethylaminoethoxy side chain, was inactive; however, the chlorinated counterpart, clomiphene, facilitated membrane translocation of PKC-ϵ (Fig. 4).

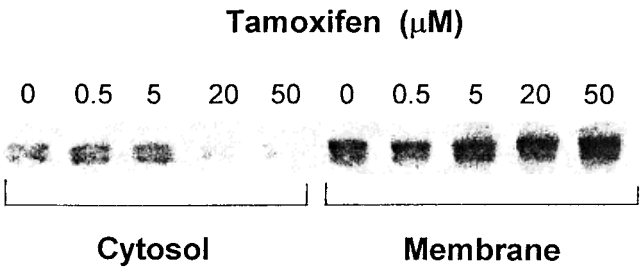

FIGURE 2 – Influence of increasing concentrations of tamoxifen on PKC-ϵ cytosol to membrane translocation in MCF-7 cells. Cells were treated with the indicated concentrations of tamoxifen for 20 min. Cytosolic and membrane fractions were analyzed for PKC-ϵ by Western blotting.

Early studies have demonstrated that the PKC activators TPA and diacylglycerol induce MCF-7 cell death (Issandou *et al.*, 1988; De Vente *et al.*, 1995). It was further hypothesized that PKC has a negative regulatory role on MCF-7 cell proliferation. Figure 5 shows the effect of tamoxifen and tamoxifen analogs on cell growth. Tamoxifen, 3-OH-tamoxifen and clomiphene, all shown to induce PKC-ϵ translocation (Fig. 4), also induced a dose-dependent reduction in cell survival (Fig. 5). 3-OH-tamoxifen, at a concentration of 5.0 μM, inhibited cell growth by 90% (Fig. 5), and this correlated with the strong effect on PKC-ϵ translocation (Fig. 4). The IC_{50} for tamoxifen and 3-OH-tamoxifen was in the range of 3 μM, whereas the IC_{50} for clomiphene was approximately 1.0 μM. In contrast, triphenylethylene and diethyltamoxifen, both inactive with respect to PKC-ϵ translocation, were also inactive with regard to influence on MCF-7 cell growth (Fig. 5).

Clomiphene elicited PKC-ϵ translocation (Fig. 4) and was the most cytotoxic of the agents tested (Fig. 5). To gain information regarding the type of cell death caused by the cytotoxic triphenylethylenes, we analyzed DNA in cells exposed to clomiphene. As shown in Figure 6, agarose gel analysis of DNA revealed classical oligonucleosomal DNA fragmentation (Fig. 6, lane 3), a characteristic of apoptosis. DNA analysis in untreated controls (Fig. 6, lane 2) displayed no DNA ladder pattern. These results strongly favor an apoptotic as opposed to necrotic mechanism of cell death.

DISCUSSION

The mechanism for the antiproliferative activity of tamoxifen on various cells, when estrogen receptors are not involved, is poorly understood. In this work, we demonstrate specific translocation of PKC-ϵ from cytosolic to particulate compartments of MCF-7 cells treated with tamoxifen or several biologically reactive triphenyl-

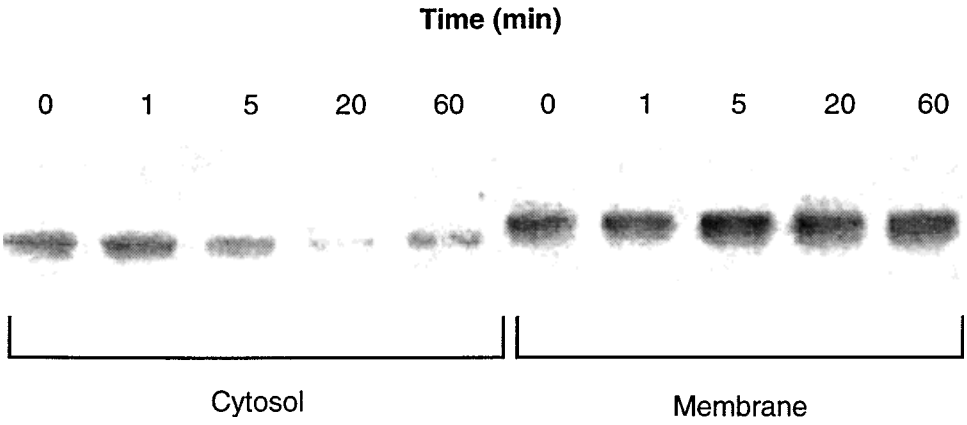

FIGURE 3 – Time course for tamoxifen-induced PKC-ϵ translocation to membranes in MCF-7 cells. Cells were incubated with 20 μM tamoxifen for the indicated times, fractionated and analyzed for PKC-ϵ by Western blotting.

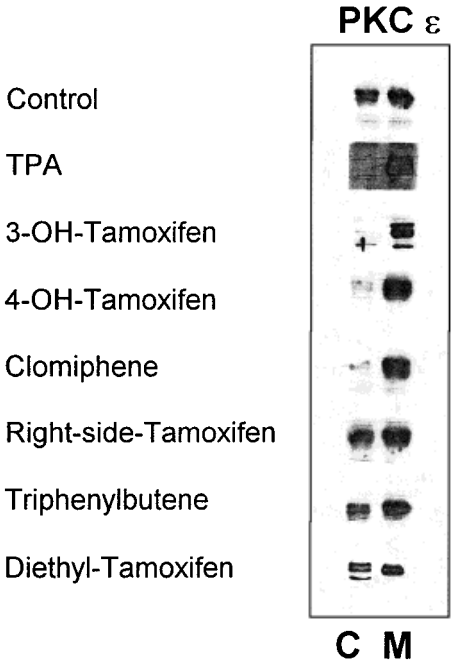

FIGURE 4 – Structure-activity relationship of tamoxifen analogs for translocation of PKC-ϵ in MCF-7 cells. Cells were incubated for 20 min with 80 nM of TPA or with 20 μM of each of the indicated agents. PKC-ϵ translocation was analyzed as described in Material and Methods. C, cytosol; M, membranes.

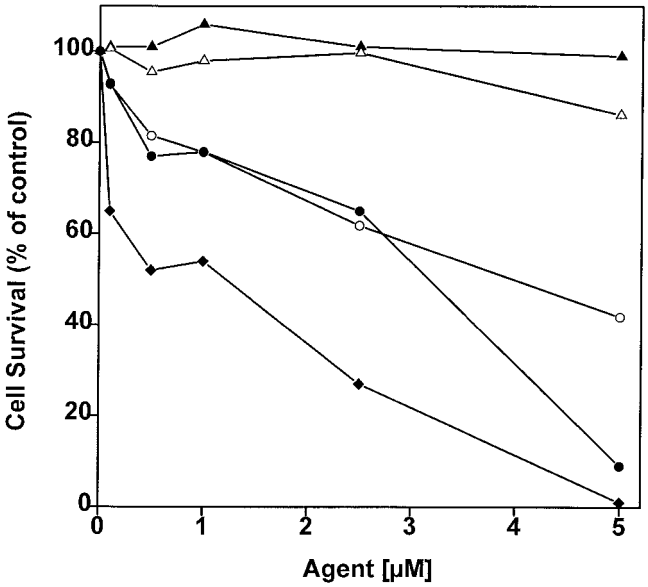

FIGURE 5 – Relationship between triphenylethylene-activated PKC-ϵ and MCF-7 cell viability. Cells were exposed to 0–5.0 μM of each of the indicated drugs for 72 hr. Cell viability was determined using the cell proliferation assay described in Material and Methods. Each point represents the mean of 6 replicate determinations from a representative experiment, and the standard deviation was <10% of the mean. (▲) Triphenylethylene; (△) diethyltamoxifen; (●) 3-OH-tamoxifen; (○) tamoxifen; (♦) clomiphene.

ethylene analogs. The type of cell death elicited by the most cytotoxic analog studied, clomiphene, was shown to be apoptosis. Signaling pathways that induce apoptosis are cell type-specific. Whereas activation of PKC in some cells inhibits apoptosis, the opposite has been demonstrated in other cell types (reviewed in De Vente *et al.,* 1995). Further, the capacity of tamoxifen to induce programmed cell death in ER⁻ cancers (Gelmann, 1996) underscores the importance of continued research on mechanisms of triphenylethylene action.

Previously, we reported on the structure-activity relationship of tamoxifen and analogs for activation of phospholipase D (Cabot *et al.,* 1997). In that work we demonstrated that hydroxylated agents such as 4-OH-tamoxifen were poor phospholipase D agonists, whereas in the present study hydroxylated analogs were reactive with regard to PKC translocation. These divergent effects are likely related to the different cell types used in the experiments. Although it was shown that activation or phospholipase D by tamoxifen was a PKC-related response, the specific PKC isozyme was not identified.

There is controversy surrounding the antagonist/agonist influence of tamoxifen on PKC. Earlier studies demonstrated that tamoxifen inhibited PKC (O'Brian *et al.,* 1985; Issandou *et al.,* 1990; Horgan *et al.,* 1986), and proposed that such action was central to the antitumor activity. In one instance, tamoxifen did not inhibit the catalytic action of PKC but reduced binding of phorbol dibutyrate in a cell-free assay (O'Brian *et al.,* 1985). In another example, using intact neutrophils, tamoxifen inhibited phorbol ester-stimulated oxidase activation (Horgan *et al.,* 1986). These early experiments were conducted prior to knowledge of PKC isozymes. More recent studies have changed our view of triphenylethylene interaction with PKC. PKC activation by triphenylethylenes has been demonstrated *in vitro* and in intact cells (Bignon *et al.,* 1991). Tamoxifen can be inhibitory for PKC when assayed in cell-free systems (O'Brian *et al.,* 1985), but has an opposite influence in intact cells (Issandou *et al.,* 1990). This raises the question: Is tamoxifen antiproliferative because it inhibits PKC? Activation of PKC is usually associated with a mitogenic response. However, in human breast cancer cells it has been amply demonstrated that TPA and diacylglycerol, activators of PKC, inhibit cell growth (Issandou *et al.,* 1988; De Vente *et al.,* 1995). The influence of tamoxifen on PKC-ϵ may designate a link between drug site of action and antiproliferative activities in breast cancer, especially in the ER⁻ setting.

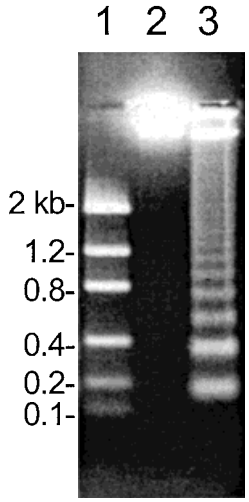

FIGURE 6 – Clomiphene treatment of MCF-7 cells elicits apoptosis. Cells were seeded in 10 cm dishes (250,000 cells/dish) and treated the following day with clomiphene (15 μM) for 72 hr. Cellular DNA was analyzed by agarose gel electrophoresis as described in Material and Methods. Lane 1, low molecular weight DNA mass ladder standard; lane 2, control cells; lane 3, clomiphene-treated cells.

ACKNOWLEDGMENTS

This work was supported by the Breast Cancer Research Program of the University of California (0211) (M.C.C.), Ben B. and Joyce E. Eisenberg Foundation, Fashion Footwear Association of New York (FFANY) Shoes on Sale® and Cancer Research Campaign, U.K. (M.J., I.R.H.).

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