

Inhibition of phorbol ester-stimulated phospholipase D activity by chronic tamoxifen treatment in breast cancer cells

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Abstract We have shown that in an estrogen receptor-negative multidrug-resistant subline of MCF-7 human breast carcinoma cells longer-term (24 h), but not shorter-term (30 min), treatments with clinically relevant (2–5 μ M) concentrations of tamoxifen (TAM) inhibited phorbol ester-stimulated phospholipase D (PLD) activity by 50–80%. TAM caused these inhibitory effects without inducing membrane translocation or down-regulation of protein kinase C- α , the major mediator of phorbol ester effects on PLD activation. The results raise the possibility that prolonged inhibition of the protein kinase C- α -regulated PLD system may contribute to the cytotoxic effects of tamoxifen in estrogen receptor-negative breast cancer cells.

Key words: Phospholipase D activity inhibition; Tamoxifen

1. Introduction

Tamoxifen (TAM), a non-steroidal anti-estrogen, has been used widely and effectively to treat hormone-responsive breast cancer [1–3]. In cell culture studies, the inhibitory effects of TAM on cell growth can involve both estrogen receptor (ER)-dependent and ER-independent components. Generally, doses of TAM below 1 μ M are cytostatic reflecting inhibition of ER function, while 1–10 μ M concentrations of TAM seem to induce cytotoxicity independently of the ER status [4,5]. In both ER-positive and -negative cells induction of apoptosis may be responsible for the cytotoxic effects of TAM [6,7].

Since ER-independent inhibition of cell growth can be achieved by clinically relevant concentrations of TAM, this drug might also have beneficial effects in patients with ER-negative tumors [8]. For this reason, it would be important to determine the mechanism(s) involved in the cytotoxic effects of TAM in ER-negative breast cancer cells.

Human breast tumors contain elevated levels of protein kinase C (PKC) activity [9]. Activation of PKC by phorbol 12-myristate 13-acetate (PMA) or 1,2-diacylglycerol can lead to increased phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho) and/or phosphatidylethanolamine (PtdEtn) (reviewed in [10]). In breast cancer cells the major regulator of PtdEtn hydrolysis is PKC- α , while stimulation of PtdCho hydrolysis by PMA may involve both PKC- α and PKC- β I [11]. The PKC-regulated PLD system is likely to be an important component of the signal transduction sys-

tem [10]. Since higher concentrations of TAM have been shown to inhibit PKC activity in vitro [12–14], we examined possible inhibition of PMA-stimulated PLD activity by TAM. For this purpose, an ER-negative multidrug-resistant subline of MCF-7 human breast carcinoma cells (MCF-7/MDR cells) and NIH 3T3 fibroblasts were chosen, because in these cells PLD activity is well characterized [15,16], including the short-term stimulatory effects of TAM on PtdEtn and PtdCho hydrolysis [17]. The results show that longer-term (24 h), but not shorter-term (30 min), treatments with TAM inhibit PMA-induced PLD activity.

2. Materials and methods

2.1. Materials

TAM, PMA and Dowex-50W(H⁺) were purchased from Sigma; [2-¹⁴C]ethanolamine (55 mCi/mmol) and [methyl-¹⁴C]choline (55 mCi/mmol) were bought from Amersham; tissue culture reagents were purchased from Gibco-BRL; and the polyclonal antibody raised against PKC- α was kindly provided by Dr. Yusuf A. Hannun (Duke University, Durham, NC, USA).

2.2. Cell culture

MCF-7/MDR cells, generously provided by Dr. Kenneth Cowan (National Cancer Institute, NIH, Bethesda, MD, USA), were maintained in Richter's modified Eagle medium supplemented with 2 mM glutamine, 12 mg/ml L-proline, 50 μ g/ml gentamicin and 10% fetal bovine serum. NIH 3T3 clone 7 fibroblasts, obtained from Dr. Douglas R. Lowy (National Cancer Institute, NIH, Bethesda, MD, USA), were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, penicillin (50 U/ml), streptomycin (50 μ g/ml) and glutamine (2 mM).

2.3. Western blot analysis of PKC- α

MCF-7/MDR cells in 60 mm diameter dishes were incubated in the absence or presence of 5–25 μ M TAM or 100–300 nM PMA for 20 min or 24 h. Then the cells were rapidly scraped into 1.5 ml homogenization buffer containing 20 mM Tris-HCl pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 100 μ g/ml leupeptin and 25 μ g/ml aprotinin. After homogenization, homogenates were centrifuged at 15 000 \times g for 20 min to prepare cytosolic and particulate fractions. The fractions (35–40 μ g protein each) were subjected to SDS-PAGE (10% acrylamide minigel) and proteins were transblotted from gels to nitrocellulose membranes. The membranes were reacted with polyclonal antibody to PKC- α (used at 1:1000 dilution), and the immunoreactive proteins were stained and analyzed as described earlier [18].

2.4. Determination of PLD-mediated formation of [¹⁴C]ethanolamine and [¹⁴C]choline from prelabeled phospholipids

MCF-7/MDR cells or NIH 3T3 fibroblasts, grown in 12-well plates, were labeled with [¹⁴C]ethanolamine (0.4 μ Ci/ml) or [¹⁴C]choline (0.6 μ Ci/ml) for 48 h; when appropriate, 2–5 μ M TAM or 300 nM PMA was present during the last 21 h of the incubation period. At the end of the labeling period the cell cultures were confluent. The cells were washed and then incubated for 3 h in fresh medium to complete incorporation of remaining radioactive precursors into cellular phospholipids; when appropriate, TAM or PMA was added back to the medium to complete the 24 h treatment schedule. Then washed cells were treated with PMA or TAM for 30 min or 90 min in the presence

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Abbreviations: TAM, tamoxifen; ER, estrogen receptor; PKC, protein kinase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PMA, phorbol 12-myristate 13-acetate

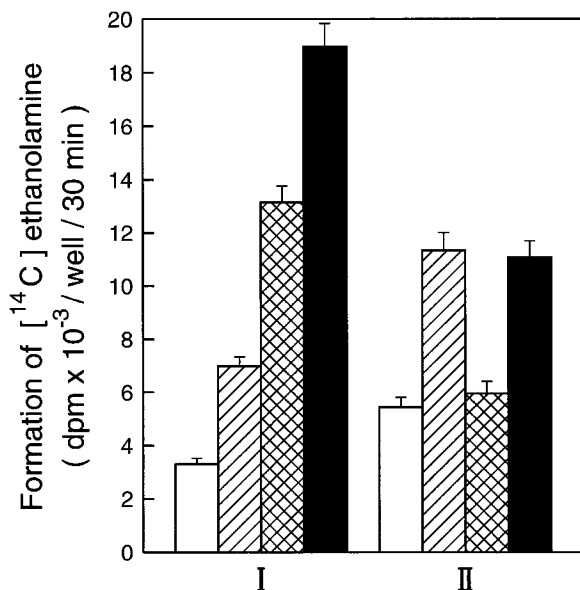


Fig. 1. Combined stimulatory effects of TAM and PMA on PtdEtn hydrolysis in MCF-7/MDR cells. Cells were labeled with [¹⁴C]ethanolamine for 48 h, and were untreated (I) or treated (II) with 300 nM PMA for the last 24 h of the labeling period. Washed cells were incubated for 30 min in the absence (empty columns) or presence of 25 μM TAM (hatched columns), 100 nM PMA (cross-hatched columns), or TAM plus PMA (black columns). The ¹⁴C content of PtdEtn was 166 000 dpm/well. Data represent the mean ± S.E.M. of eight incubations performed in two separate experiments with the same passage of cells. Similar results were obtained in a third experiment performed in quadruplicate.

of 2 mM ethanolamine or 20 mM choline, as appropriate, to prevent metabolism of radiolabeled products. Incubations were terminated by scraping the cells into 2 ml ice-cold methanol, followed by rapid transfer of the methanol extract to 2 ml of chloroform. After phase separation, [¹⁴C]ethanolamine, [¹⁴C]choline, and their metabolites were separated on Dowex-50W(H⁺)-packed columns as described earlier [19].

3. Results and discussion

The first goal was to determine the possible role of PKC in the short-term stimulatory effects of TAM on PLD activity [17]. Since in an ER-negative breast cancer cell line TAM was shown to increase the activity of an unspecified calcium-dependent PKC isozyme(s) [20], stimulation of PLD by TAM could be mediated by PKC. However, TAM also was shown to inhibit the activity of calcium-dependent PKC isozyme(s) in vitro [12–14]; thus, in principle, TAM could also inhibit PMA-induced activation of PLD. Finally, if PKC-α, a major regulator of PLD in breast cancer cells and fibroblasts [11], is neither stimulated nor inhibited by TAM, then TAM would not be expected to modulate stimulation of PLD activity by PMA. With these options in mind, first we determined the combined short-term effects of TAM and PMA on PLD activity in MCF-7/MDR cells. In these cells, activated PLD hydrolyzes mainly PtdEtn [16]. This specific PLD activity can be determined in [¹⁴C]ethanolamine-labeled cells where PMA- or TAM-induced formation of [¹⁴C]ethanolamine from the prelabeled cellular pool of PtdEtn occurs by a PLD-mediated mechanism [15–17]. As shown in Fig. 1-I, in [¹⁴C]ethanolamine-labeled MCF-7/MDR cells the effects of maximally effective concentrations of TAM (25 μM) and

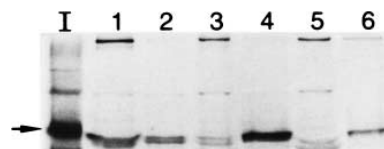


Fig. 2. Western blot analysis of the shorter-term and longer-term effects of PMA on the cellular status of PKC-α. MCF-7/MDR cells were untreated (lanes 1, 2) or were treated with 100 nM PMA for 20 min (lanes 3, 4), or with 300 nM PMA for 24 h (lanes 5, 6). Lane I represents PKC-α standard from rat brain shown by the arrow. Lanes 1, 3, 5 were loaded with 40 μg of soluble fraction protein and lanes 2, 4, 6 were loaded with 40 μg of particulate fraction protein. This experiment was repeated twice with similar results.

PMA (100 nM) on the formation of [¹⁴C]ethanolamine were additive.

Although the above data suggested that the stimulatory effect of TAM on PLD activity was not mediated by PKC-α, we examined this issue further. As shown in Fig. 2, a treatment with 100 nM PMA for 20 min caused membrane translocation of PKC-α, while a treatment with 300 nM PMA for 24 h decreased the cellular content of this isozyme about 75%. (A similar chronic treatment with 100 nM PMA caused less reduction in cellular PKC-α.) A prolonged treatment with 300 nM PMA slightly enhanced PLD activity, but PLD activity was not further enhanced by newly added PMA. These observations probably reflect that the remaining cellular pool of PKC-α after chronic PMA treatment was fully activated and functional with respect to its ability to activate PLD. Most importantly, the stimulatory effect of TAM on

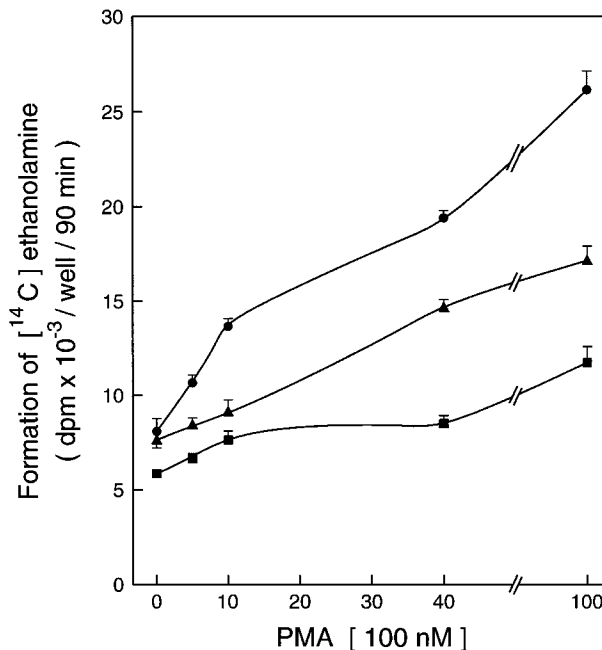


Fig. 3. Longer-term treatments with TAM inhibit PMA-stimulated hydrolysis of PtdEtn in MCF-7/MDR cells. Cells were labeled with [¹⁴C]ethanolamine for 48 h, and were untreated (●) or treated with 2 μM TAM (▲), or 5 μM TAM (■) for the last 24 h of the labeling period. Washed cells were incubated, in the absence of TAM, with 0–100 nM concentrations of PMA for 90 min. Each point represents the mean ± S.E.M. of eight incubations performed in two separate experiments with the same passage of cells. Similar results were obtained in two other experiments each performed in triplicate.

PtdEtn hydrolysis was not affected by the down-regulation of a major fraction of cellular PKC- α (Fig. 1-II). In NIH 3T3 fibroblasts as well, TAM (25 μ M) and PMA (100 nM) had additive effects on the hydrolysis of both PtdCho and PtdEtn, and down-regulation of PKC- α by chronic (24 h) treatment with 300 nM PMA failed to modify the stimulatory effects of TAM on these PLD activities (data not shown). These data indicated that short-term use of TAM does not prevent regulation of PLD by PKC- α , and that the transient stimulatory effect of TAM on PLD activity [17] is not mediated by PKC- α .

In contrast to short-term treatments, longer-term (24 h) treatments of MCF-7/MDR cells with 2 and 5 μ M TAM inhibited the effects of PMA on PtdEtn hydrolysis by about 50 and 80%, respectively (Fig. 3). It should be noted that longer-term treatments with these concentrations of TAM had no significant effects on the cellular level of [14 C]PtdEtn, reflecting the transient nature of the stimulatory effect of TAM on PLD activity. However, 10–15 μ M concentrations of TAM decreased both the cell numbers and the cellular level of PtdEtn after treatments for 24 h; for this reason, we have not attempted to increase the concentration of TAM above 5 μ M.

A longer-term (24 h) treatment of NIH 3T3 fibroblasts with 5 μ M TAM inhibited the stimulatory effect of PMA on PtdCho hydrolysis about 50% (Fig. 4A); PMA-induced hydrolysis of PtdEtn was less affected (\sim 35% inhibition; Fig. 4B). Overall, 5 μ M TAM was a somewhat less effective inhibitor of PMA-induced PLD activity in fibroblasts than in MCF-7/MDR cells. The same is true when the effects of a lower (2 μ M) concentration TAM are compared in fibroblasts and MCF-7/MDR cells (Fig. 4 compared to Fig. 3).

Recently, Gundimeda et al. [20] reported that longer-term

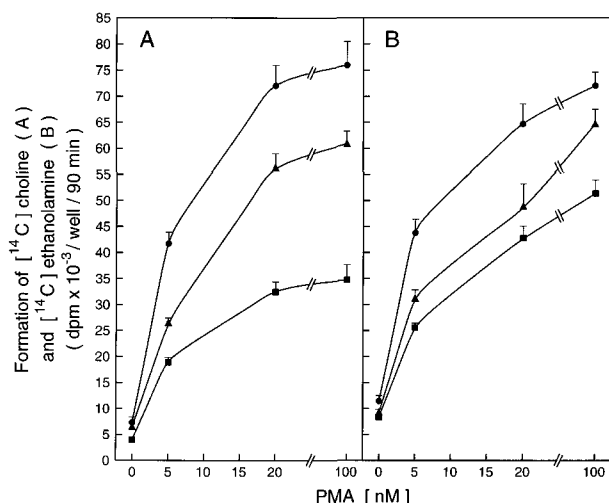


Fig. 4. Effects of longer-term treatments with TAM on PMA-stimulated phospholipid hydrolysis in NIH 3T3 fibroblasts. Fibroblasts were labeled with [14 C]choline (A) or [14 C]ethanolamine (B) for 48 h, and were untreated (●) or treated with 2 μ M TAM (▲) or 5 μ M TAM (■) for the last 24 h of the labeling period. Washed cells were incubated, in the absence of TAM, with 0–100 nM concentrations of PMA for 90 min. The [14 C] content of PtdCho and PtdEtn was 228 000 and 239 000 dpm/well, respectively. Each point represents the mean \pm S.E.M. of six incubations in two separate experiments with the same passage of cells. Similar results were obtained in a third experiment performed in quadruplicate.

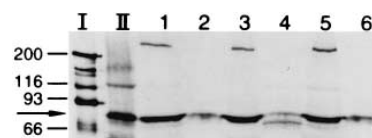


Fig. 5. Western blot analysis of PKC- α in TAM-treated MCF-7/MDR cells. MCF-7/MDR cells were untreated (lanes 1, 2) or were treated with 5 μ M TAM for 20 min (lanes 3, 4) or 24 h (lanes 5, 6). Lane I represents molecular weight standards. Lane II represents PKC- α standard from rat brain shown by the arrow. Lanes 1, 3, 5 were loaded with 35 μ g soluble fraction protein and lanes 2, 4, 6 were loaded with 35 μ g of particulate fraction protein. This experiment was repeated once with similar results.

treatments of MDA-MB-231 breast carcinoma cells with TAM lead to the down-regulation of calcium-dependent PKC activity. Our results also indicated that the function of PKC- α as a regulator of PLD activity in MCF-7/MDR cells is blocked by extended treatments with TAM. It was of interest to see if such TAM-induced reduction in the function of PKC- α is due to its physical down-regulation perhaps preceded by membrane translocation. However, in MCF-7/MDR cells 5 μ M TAM induced neither membrane translocation nor down-regulation of PKC- α after treatments for 20 min or 24 h (Fig. 5). In other experiments, treatments with 15 and 25 μ M TAM for 20 min also failed to induce membrane translocation of PKC- α (data not shown).

In treated patients the intratumor concentration of TAM can reach a level equivalent to 3.4–6.7 μ M [21]. Here, we have shown that chronic treatments with 2–5 μ M concentrations of TAM can cause significant inhibition of PMA-stimulated PLD activity particularly in MCF-7/MDR cells. These observations suggest that the observed inhibition of this important regulatory function of PKC- α may be a clinically relevant effect of TAM perhaps contributing to the hitherto unexplained beneficial effects of TAM in patients with ER-negative tumors [8].

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