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Tamoxifen stimulates phospholipase D activity by an estrogen receptor-independent mechanism

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Abstract The effects of tamoxifen (TAM), a widely used agent in the treatment of breast cancer, were examined on phospholipase D (PLD)-mediated phospholipid hydrolysis. In drug-sensitive MCF-7 human breast carcinoma cells TAM, similar to several well-established activators of PLD, had no effect on phospholipid hydrolysis. In an estrogen receptor-deficient multidrug-resistant subline of MCF-7 cells, TAM preferentially stimulated the hydrolysis of phosphatidylethanolamine; two-fold stimulation required 2.5 or 5 μM TAM in the absence or presence of serum, respectively. In NIH 3T3 fibroblasts significant (4- to 4.8-fold) stimulation of phosphatidylethanolamine and phosphatidylcholine hydrolysis in the presence of serum required 10 μM TAM. These data establish that TAM can stimulate PLD activity by an estrogen receptor-independent mechanism.

Key words: Tamoxifen; Phospholipase D; Breast cancer; Fibroblast

1. Introduction

Tamoxifen (TAM), a triphenylethylene non-steroidal anti-estrogen, has been used widely and effectively for treatment of patients with all stages of breast cancer [1–3]. While the response rate of estrogen receptor-positive tumors to TAM is higher, a relatively high percentage of estrogen receptor-negative patients also demonstrate a response to tamoxifen [3]. This indicates that TAM cannot be regarded as a simple anti-estrogen, which is an important consideration because development of initially estrogen-dependent non-metastatic breast tumors to a more aggressive estrogen-independent phenotype is almost inevitable. While the mechanism(s) for the estrogen-independent growth inhibitory effect(s) of TAM is unknown, TAM has been shown to exert estrogen receptor-independent effects on the function of calmodulin [4–6] and protein kinase C (PKC) [7–9]. However, these latter effects required relatively high concentrations of TAM which appear to be physiologically irrelevant. Therefore, we have examined possible effects of TAM on some other components of the signal transduction system involved in growth regulation.

The hydrolysis of phospholipids by hormone- and phorbol ester-stimulated phospholipase D (PLD) results in the formation of phosphatidic acid [10,11]. Phosphatidic acid can serve as a messenger molecule with the potential to regulate cell growth [12–17], metastasis of tumor cells [18], exocytosis [19–24], as well as other cellular processes (reviewed in [25]). Considering the emerging role of PLD in signal transduction as well as the known ability of TAM to alter the physical properties of biological membranes [26,27], the goal of this work was to examine possible modification of PLD activity by TAM. TAM was found to stimulate the hydrolysis of both phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) in NIH 3T3 fibroblasts and in a multidrug-resistant subline of MCF-7 human breast carcinoma cells, but not in the parent MCF-7 cells, by an estrogen receptor-independent mechanism. The possible relevance of these findings to the effects of TAM on multidrug resistance and tumor growth will be discussed.

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

2. Materials and methods

2.1. Materials

TAM, phorbol 12-myristate 13-acetate (PMA), and Dowex-50W(H^+) were purchased from Sigma; [methyl-^{14}C]choline chloride (50 mCi/mmol), [2-^{14}C]ethanolamine (50 mCi/mmol) and [1-^{14}C]palmitic acid (60 mCi/mmol) were from Amersham; phosphatidylethanol (PtdEtOH) was from Avanti Polar Lipids Inc., and tissue culture reagents were purchased from Gibco.

2.2. Cell culture

The MCF-7 human breast cancer cell lines (drug-sensitive MCF-7/WT cells and multidrug-resistant MCF-7/MDR cells) were generously provided by Dr. Kenneth Cowan (National Cancer Institute, NIH, Bethesda, MD, USA). MCF-7 cells were maintained as described earlier [28]. NIH 3T3 clone 7 fibroblasts were obtained from Dr. Douglas R. Lowy (National Cancer Institute, Bethesda, MD, USA) and were cultured continuously in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and glutamine (2 mM).

2.3. Measurement of PtdEtOH formation in MCF-7 and NIH 3T3 cells

Cells were grown in 6-well tissue culture dishes for 48 h to about 70–80% confluency and labeled with [1-^{14}C]palmitic acid (0.3 μCi/ml) for the last 24 h. Cells were washed, incubated in fresh medium for an additional 2 h (to lower the amount of unesterified labeled palmitic acid) and then treated with TAM and/or PMA in complete medium in the presence of 150 mM ethanol for 30 min at 37°C. Incubations were terminated by scraping the cells into 2 ml of ice-cold methanol, followed by rapid transfer of the methanol extract to 2 ml of chloroform. PtdEtOH was separated from other phospholipids on potassium oxalate (1%)-impregnated Silica gel H plates (Analtech) with a solvent system of chloroform/methanol/acetone/acetic acid/water (50:10:15:10:2, by vol).

2.4. Labeling of cellular phospholipids with [^{14}C]ethanolamine and [^{14}C]choline

MCF-7 and NIH 3T3 cells were seeded in 150 mm-diameter plastic dishes and were incubated with either [2-^{14}C]ethanolamine (0.25 μCi/ml) or [methyl-^{14}C]choline (0.35 μCi/ml) for 48 h. Cells were washed twice and then incubated in the corresponding fresh medium for 3 h; this additional incubation step reduces the cellular levels of water-soluble ^{14}C-labeled precursors, which are also the products of PLD action, by a factor of 3- to 5-fold [29]. Cells were harvested by gentle scraping from 3–6 dishes. After an additional 20 min incubation period

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(allowed for the return of slightly elevated 1,2-diacylglycerol levels to normal levels [30]), cells were pelleted for 7 min at 500 × *g*. Cells were resuspended in either complete medium (Figs. 1 and 2) or in serum-free medium (Fig. 3) and then treated with TAM in the presence of 2 mM ethanolamine or 20 mM choline, as appropriate; these latter compounds served to prevent phosphorylation of newly formed [^{14}C]ethanolamine and [^{14}C]choline [29,31]. Suspended and attached MCF-7/MDR [28] and NIH 3T3 cells [31,32] were similarly responsive to PMA, sphingosine and H_2O_2 with respect to the activation of PLD, but suspended cells contained 5–10 times less background levels of ^{14}C-labeled precursors. This made evaluation of the effects of lower concentrations of TAM on phospholipid hydrolysis possible.

2.5. Determination of water-soluble products of phospholipid hydrolysis

Fractionation of ethanolamine and choline metabolites was performed on Dowex-50W(H^+)-packed columns (Bio-Rad Econo columns; 1 ml bed volume) as previously described [32].

3. Results

Determination of the formation of [^{14}C]ethanolamine and [^{14}C]choline from the respective prelabeled phospholipids represents a more sensitive assay of PLD activity compared to determination of PtdEtOH formation. For this reason, the possible effects of different concentrations of TAM on PtdEtn and PtdCho hydrolysis were first determined in [^{14}C]ethanolamine- and [^{14}C]choline-labeled MCF-7 cells. As was previously described in detail [28], MCF-7/WT cells exhibited only a low level of PLD activity which was not enhanced by PMA, sphingosine or H_2O_2. Presently, TAM (2.5–50 μM) also failed to enhance PLD-mediated hydrolysis of either [^{14}C]PtdEtn or [^{14}C]PtdCho in these cells (data not shown).

In contrast, MCF-7/MDR cells contain a PLD system which preferentially hydrolyzes PtdEtn in the presence of PMA and other activators [28]. Similarly, treatment of these cells with 5–50 μM concentrations of TAM in a 10% serum-containing medium caused preferential stimulation of [^{14}C]PtdEtn hydrolysis during either a 20 min (Fig. 1A) or 40 min (Fig. 1B) incubation period. Well detectable (1.45- to 1.7-fold) stimulation of PtdEtn hydrolysis in this complete system was observed with 5 μM TAM, while maximal (4.25-fold) stimulation required 50 μM TAM.

NIH 3T3 fibroblasts contain PLD systems which hydrolyze both PtdEtn and PtdCho with similar efficiencies in response to PMA [31,32], sphingosine [33] and H_2O_2 [34]. Treatment of [^{14}C]ethanolamine- and [^{14}C]choline-labeled NIH 3T3 fibroblasts with 10 μM TAM in 10% serum-containing medium elicited similar (1.6- to 1.65-fold) stimulatory effects on the hydrolysis of [^{14}C]PtdEtn and [^{14}C]PtdCho (Fig. 2). However, higher (15–50 μM) concentrations of TAM preferentially stimulated the hydrolysis of PtdCho, so that 50 μM TAM stimulated the hydrolysis of PtdCho and PtdEtn 11.5- and 4.2-fold, respectively (Fig. 2).

It is well known that TAM binds to serum proteins, thus, the concentration of serum in culture medium significantly affects the concentration-dependent inhibitory effects of TAM on cell growth [35,36]. For this reason, it appeared important to test the effect of serum on TAM-induced phospholipid hydrolysis. When PLD assays with MCF-7/MDR cells were performed in the absence of serum, TAM at a concentration as low as 2.5 μM stimulated the hydrolysis of [^{14}C]PtdEtn 1.8-fold (Fig. 3A). However, at higher concentrations, TAM had similar effects on PtdEtn hydrolysis in the absence or presence of 2 or 5% serum (Fig. 3A). It should be noted that these latter effects of TAM were also very similar to those observed in the presence of 10% serum (Fig. 1). In contrast, in NIH 3T3 fibroblasts 2.5–50 μM concentrations of TAM had only small or no effects on [^{14}C]PtdEtn hydrolysis when the assays were performed in the absence or presence of 2% serum (Fig. 3B). However, in the

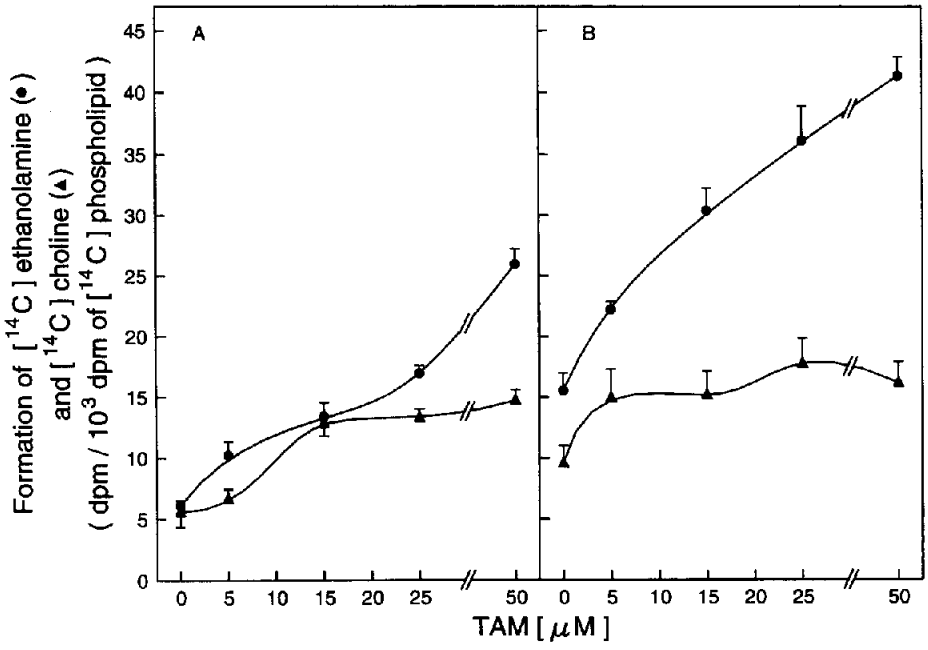

Fig. 1. Concentration- and time-dependent effects of TAM on phospholipid hydrolysis in MCF-7/MDR cells. Cells were labeled with [^{14}C]ethanolamine (●) or [^{14}C]choline (▲) for 48 h, followed by treatment of suspended cells with varying concentrations (2.5–50 μM) of TAM for 20 min (A) or 40 min (B). In each case, the incubation medium contained 10% serum. The average ^{14}C content of PtdEtn and PtdCho was 876,000 and 665,000 dpm/10^6 cells, respectively. Each point represents the mean ± S.E.M. of four independent incubations. Similar results were obtained in two other experiments, each performed in triplicate.

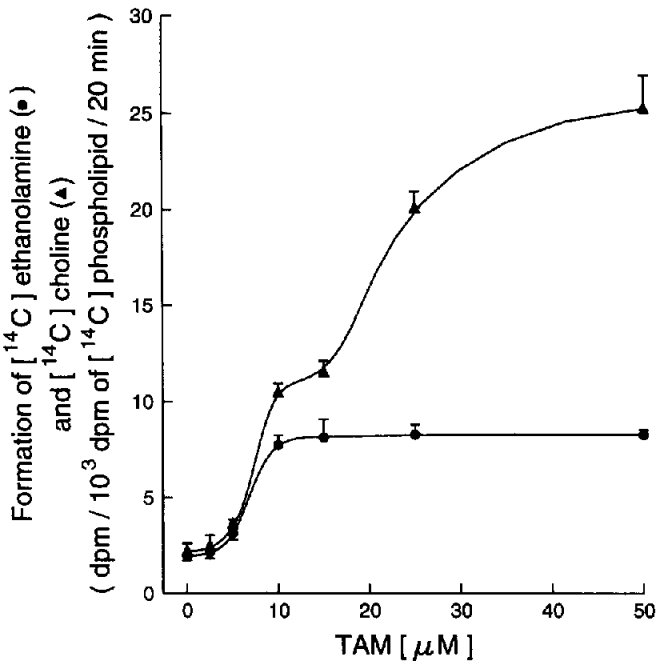

Fig. 2. Concentration-dependent effects of TAM on phospholipid hydrolysis in NIH 3T3 fibroblasts. Cells were labeled with [^{14}C]ethanolamine (●) or [^{14}C]choline (▲) for 48 h followed by treatment of suspended cells with 2.5–50 μM concentrations of TAM for 20 min. In each case, the incubation medium contained 10% serum. The average ^{14}C content of PtdEtn and PtdCho was 1.13×10^6 and 855,000 dpm/10^6 cells, respectively. Each point represents the mean ± S.E.M. of four independent incubations. Similar results were obtained in three other experiments, each performed in triplicate.

presence of 5% serum (Fig. 3B), the stimulatory effects of TAM on [^{14}C]PtdEtn hydrolysis were similar, although still of a somewhat lesser extent, than those observed in the presence of 10% serum (Fig. 2). In fibroblasts, significant stimulation of PtdCho hydrolysis by TAM also required the presence of serum (data not shown).

To confirm that TAM-induced phospholipid hydrolysis was mediated by PLD, the effect of TAM on the formation of PtdEtOH, which is a specific function of PLD, was also determined. As expected, in MCF-7/WT cells 25 μM TAM had no effect on PtdEtOH formation, while this drug significantly enhanced PtdEtOH formation both in MCF-7/MDR cells (5.9-fold stimulation) and NIH 3T3 fibroblasts (3.7-fold stimulation, Fig. 3). Although TAM is a reported inhibitor of PKC in vitro [7–9], in these experiments TAM and PMA had additive effects on PtdEtOH formation (Fig. 4).

4. Discussion

The fact that drug-sensitive breast carcinoma cells do not contain appreciable levels of functional PLD ([28], and this work) suggests that this enzyme may not be required for the aggressive growth of these cells. Consequently, in these drug-sensitive cells PLD can not mediate the growth inhibitory effect of TAM.

The development of multidrug resistance is accompanied, at least in MCF-7 cells, by increased expression of a relatively PtdEtn-specific PLD activity [28]. Considering that TAM can reverse MDR1-associated multidrug resistance [37], and that MCF-7/MDR cells are cross-resistant to TAM ([38], and references therein), the observed stimulatory effects of TAM on PLD activity are of considerable interest. Among other possi-

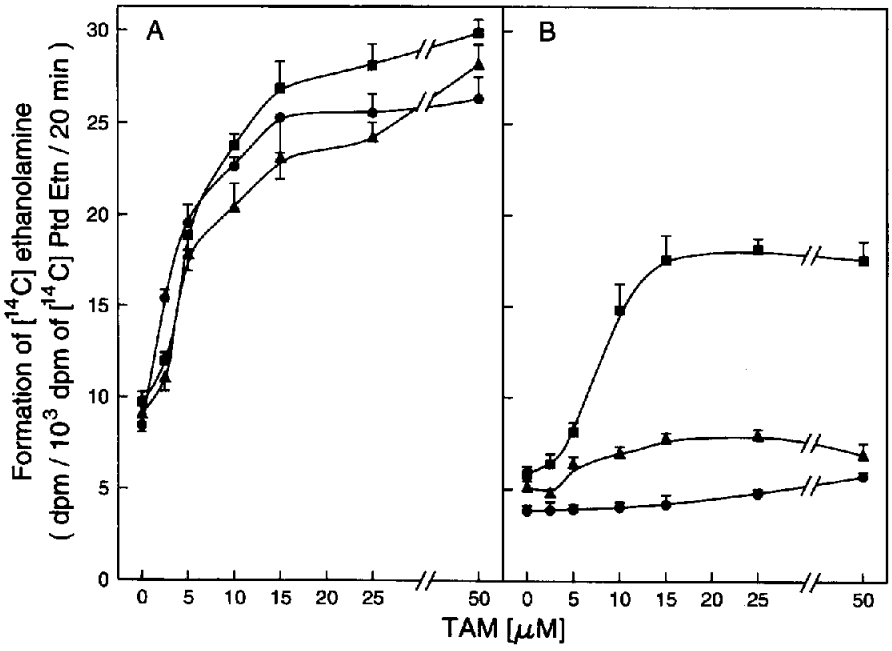

Fig. 3. Combined effects of serum and TAM on PtdEtn hydrolysis. MCF-7/MDR (A) and NIH 3T3 (B) cells were labeled with [^{14}C]ethanolamine followed by treatment of suspended cells with 2.5–50 μM concentrations of TAM for 20 min in the absence (●) or presence of 2% serum (▲) or 5% serum (■). Each point represents the mean ± S.E.M. of six independent incubations. Similar results were obtained in another experiment performed in quadruplicate.

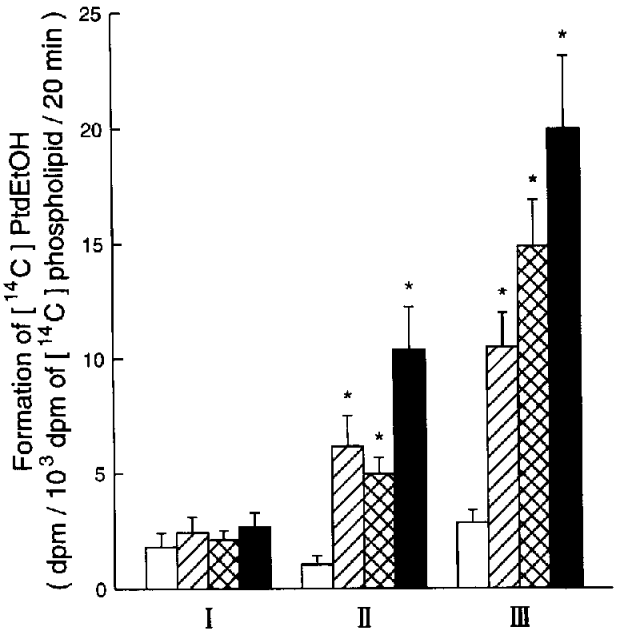

Fig. 4. Comparison of the effects of TAM and PMA on PtdEtOH formation. Attached MCF-7/WT (I), MCF-7/MDR (II) and NIH 3T3 (III) cells, prelabeled with [^{14}C]palmitic acid, were incubated for 20 min in the absence (open bars) or presence of 25 μM TAM (hatched bars), 100 nM PMA (cross-hatched bars), or TAM plus PMA (black bars). The ^{14}C content of PtdCho and PtdEtn in these cells was in the range of 512,000–876,000 and 149,000–226,000 dpm/10^6 cells, respectively. Data represent the mean ± S.E.M. of six independent incubations. *Significantly ($P < 0.01$) different from the control (no addition) value (Student't t-test).

bilities, these data suggest that TAM might be able to modify multidrug resistance by stimulating its own efflux through mechanisms involving both the PLD system and P-glycoprotein. In this regard, PtdEtn, the major substrate of PLD in MCF-7/MDR cells, has been shown to regulate the ATPase activity of P-glycoprotein [39]. It is also worth mentioning that reduced intratumoral accumulation of TAM has been found in patients with locally recurrent breast cancer [40]. Further experiments are required to prove that the effects of TAM on PLD activity and multidrug resistance are indeed causally related.

Many patients who initially respond to TAM treatment experience disease progression when taking TAM for an extended period of time. Several models have been developed, both in vitro and in vivo, to study the progression of breast cancer growth from the TAM-sensitive to the TAM-resistant stage. Because breast cancer is a highly heterogeneous disease, it is likely that multiple mechanisms will account for the TAM resistance. In this respect, it has recently been reported that while co-administration of TAM and the carcinogen 7,12-dimethylbenzanthracene to rats resulted in a dramatic reduction in the number of tumors observed, a relatively large number of TAM-treated rats still developed tumors the growth of which was dependent on TAM [41]. One cannot exclude the possibility that the observed effects of TAM on the PLD system are, in fact, part of a complex mechanism mediating a growth stimulatory, rather than a growth inhibitory, action of TAM. Another possibility is that stimulation of PLD by TAM increases the invasive capacity of resistant cells. In support of this possibility, Imamuka et al. [18] recently reported that bacterial PLD enhanced invasiveness of rat ascites hepatoma (AH130) cells. Taking into account the significance of this issue, the above discussed possible actions of TAM will certainly require further consideration.

Since MCF-7/MDR cells do not contain estrogen receptors [42,43], it is safe to conclude that the actions of TAM on the PLD system are not mediated by these receptors. Thus, it was less surprising to observe that TAM also stimulated PLD activity in fibroblasts which are not known to possess significant levels of estrogen receptors. However, while the actions of TAM in MCF-7/MDR cells did not require serum, TAM failed to significantly stimulate the PLD activity in fibroblasts in the absence of serum. The reason for these differences is not known.

It has been calculated that in treated patients the intratumor concentration of TAM can reach a level equivalent of 3.4–6.7 μM [44]. Presently, it was found that, depending on the presence of serum, 2.5–5 μM concentrations of TAM significantly stimulated PLD activity in MCF-7/MDR cells. Thus, it seems very likely that in the case of breast cancer, TAM-induced phospholipid hydrolysis represents a physiologically relevant process. In addition, the results showing that 5 μM TAM failed to induce significant PLD activation in fibroblasts suggests that drug resistant populations of breast cancer cells may be special targets for these actions of TAM.

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