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Increased Phospholipase D Activity in Multidrug Resistant Breast Cancer Cells

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SUMMARY An adriamycin-resistant MCF-7 cell line (300-fold increased resistance vs. wild type MCF-7) showed an increased phorbol ester-stimulated phospholipase D activity of approximately 4-6 times over that found in wild type cells. Phospholipase D activity was assessed by monitoring the mass of phorbol ester-induced phosphatidylethanol. The cellular phospholipase D activity was time- and phorbol ester-concentration-dependent and was obvious whether measured as an increase in the mass of PEt or in the production of ^{3}H-labeled phosphatidylethanol in cells prelabeled with [^{3}H]myristic acid. Phorbol ester also stimulated increases in the production of the mass of cellular diacylglycerol and phosphatidic acid in the adriamycin-resistant cells vs. the wild-type cells. Tests with a series of drug-resistant MCF-7 cell lines revealed a positive correlation between increased drug resistance and phorbol ester-stimulated phospholipase D activity.

Phospholipase D (PLD) plays a role in the signal-response mechanisms used by a variety of agonists including tumor-promoters, hormones, and growth factors (1,2). The activated PLD produces phosphatidic acid (PA) as a primary product, and subsequent metabolism of the PA generates diacylglycerol (DAG) (1-4). These lipid mediators are derived from membrane phospholipids other than phosphatidylinositol, primarily phosphatidylcholine (PC) (1-6) and phosphatidylethanolamine (PE) (7-9). Recent reports have described PLD activity in many different cell types using a variety of experimental conditions. However, the functional role of PLD in cellular signaling pathways is far from clear.

The overexpression of P-glycoprotein (P-gp) is associated with the development of the multidrug resistance phenotype in a variety of tumors and cell lines (10-13). P-gp is a transmembrane protein that functions as a multidrug transporter. The P-gp transporter

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ABBREVIATIONS USED: phospholipase D, PLD; 12-O-tetradecanoylphorbol-13-acetaate, TPA; phosphatidylethanol, PEt; phosphatidic acid, PA; diacylglycerol, DAG; P-glycoprotein, P-gp. thin--layer chromatography, TLC; phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylserine+phosphatidinositol+, PS/PI; sphingomyelin, SPM; wild type MCF-7 cells, WT; multidrug resitant MCF-7 cells, R300; protein kinase C, PKC; ED_{50}, dose that provides 50 % of the maximal response.

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eleminates chemotherapeutic drugs and other xenobiotics from resistant cells (10-14). Several reports suggest that substrates for the P-gp possess hydrophobic characteristics, and recent evidence and speculation suggest that substrates of P-gp can be sequestered in, and subsequently eliminated from, the membrane lipid environment of multidrug resistant cells (15). In addition, hydrophobic compounds such as steroids and dietary flavonoids can modulate the P-gp activity in cellular systems (16).

Because agonist-activated PLD can produce large changes in the lipids of cellular membranes, presumably including the plasma membrane, and because P-gp can eliminate hydrophobic compounds from membrane environments (16), we envisioned several hypothetical situations in which P-gp and PLD activities could be related. In our initial investigation of the interrelationships between PLD, lipid mediators, and P-gp, we examined the level of TPA-stimulated PLD activity in a series of multidrug resistant MCF-7 cells that were selected for resistance to adriamycin. The multidrug resistant variants overexpress P-gp, and the levels of P-gp show a positive correlation with an increasing degree of drug resistance (14). Our findings indicate the TPA-stimulated PLD activity increases as drug resistance increases, and may be related to the multidrug resistance phenotype.

METHODS AND MATERIALS Chemicals and Materials: 12-O-Tetradecanoylphorbol-13-acetate (TPA), bovine serum albumin (fatty acid free), diolein, phosphatidic acid, and adriamycin were from Sigma Chemical Company. [9,10-^{3}H]Myristic acid (39 Ci/mmol) was a product of New England Nuclear. Silica gel G thin-layer chromatography (TLC) plates were from Analtech, Inc. (Wilmington, DE). Phosphatidylethanol (PEt) was purchased from Avanti Polar Lipids (Alabaster, AL).

Cell Lines and Manipulations: the culture of wild type MCF-7 and the development of adriamycin resistant MCF-7 cell lines have been described (14). This protocol resulted in the development of three cell lines that demonstrate an IC_{50} (concentration that results in a 50% inhibition in cell growth) for adriamycin that are 65x (R65), 300x (R300), and 500x (R500) that observed in the wild type cells MCF-7 (WT). MCF-7 Cl 10.9 was established after transfection with the MDR-1 cDNA (17), and was a gift from Dr. R. Clark; National Institutes of Health, Bethesda, MD. The MCF-7 Adr cell line (18) was obtained from the laboratory of Dr. K. Cowan; National Institutes of Health, Bethesda, MD. All cell cultures were used at confluence. For experimental protocols, the cell culture medium was removed, the cells were washed once and were exposed to TPA (in fresh medium containing 0.5 mg bovine serum albumin /ml culture medium) at various concentrations or for differing time periods. When PEt was the analyte, the treatment medium contained ethanol (2%; v/v). Radiolabeling with [^{3}H]myristic acid (0.5 μCi/ml; 24 hr) was achieved as previously described (5,6).

Analyses: At the termination of the experiments, the medium was removed and the cells were washed and fixed with methanol. The cells were scraped and the lipids extracted (19). Radiolabeled lipids (and comigrating standards) were visualized by exposure to iodine vapor, removed from the TLC plate, and quantitated by scintillation counting (5,6). Mass measurements of unlabeled PEt, DAG, and PA were determined by photodensitometry of acid-charred spots on TLC plates as described (20). The Mass determinations of cellular phospholipids were accomplished with photodensitometry after resolution using TLC (21). All photodensitometric assessments of lipid mass were made using the Uniscan I video densitometer manufactured by Analtech. Phosphatidylcholine (PC) levels were used to normalize lipid values obtained from the different cell lines in a manner analogous to the strategy used by other investigators (20,22,23). Values expressed as "mol %" are calculated as nmol PEt/100 nmol PC x100. The fatty acid components of the cellular PEt were determined after recovering the PEt from TLC plates, and transesterifying the PEt using Meth Prep II (Alltech). The resultant fatty acid-methyl esters were resolved and quantitated using a Hewlett Packard GC (model 5790) interfaced to a mass selective detector (model 5871). Reconstructed ion chromatograms (spectra collected at 70 eV) were obtained using a Hewlett Packard HP-1 column (50 M; 0.32 ID; 0.17 u) and a temperature program of: 120° C 3 min; then 5° C/min to 240° C.

RESULTS Initial experiments demonstrated an elevated TPA-stimulated PLD activity in the R300 cells when compared to the WT cells. Mass measurements showed that PEt levels rose to 9.2 (±1.3) mol % of cellular PC in R300 cells treated with TPA (30 minutes), whereas in WT cells, PEt levels reached only 2.7 (±0.26) mol % of the cellular PC. Further experiments showed that TPA stimulated a concentration-dependent increase in the production of PEt in both WT and R300 MCF-7 cells (Figure 1). The ED_{50} values for the production of PEt were 30 ng TPA/ml and 10 ng TPA/ml for WT and R300, respectively. Total PEt mass production by both cell lines was maximal at TPA concentrations of approximately 200 ng/ml. However, the R300 cells produced approximately 4-fold more PEt than did the WT cells when stimulated with 200 ng TPA/ml. Several repeated experiments demonstrated that the increased TPA-stimulated PLD activity in the R300 cells ranged between 3.5-5 fold over control values (see results presented below).

Agonist-activated PLD is known to hydrolyze cellular phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (5-9). To determine whether both the WT and R300 cells possessed similar amounts of the possible phospholipid substrates for PLD, the cellular phospholipids were quantitated by photodensitometry. These analyses found that the levels of the major phospholipids were similar in both cell types. The relative phospholipid values were as follows (listed as WT vs. R300; all values are means of four determinations): PC, 48 vs. 47%; PE, 33 vs. 28%; SPM, 8 vs. 11; PS+PI, 12 vs. 14%.

The acyl composition of the agonist-induced PEt from both WT and R300 cells was also examined. The PEt was resolved by TLC, recovered from the silica gel and transmethylated to generate fatty acid methyl esters which were then subjected to gas chromatography-mass spectrometry. These comparative analyses found only slight differences in minor fatty acids from the PEt of the agonist-stimulated WT and R300 cells (Table 1).

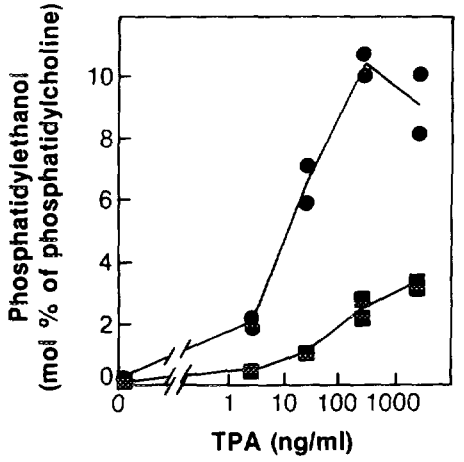

Figure 1. Concentration-dependency of Phorbol Ester-stimulated Phospholipase D activity in Wild Type and Multidrug Resistant MCF-7 Cells: Analysis of Phosphatidylethanol Mass. Cell cultures (6-well plates) of wild type (■) and multidrug resistant cells (●) were incubated for 20 min in the presence of phorbol ester (TPA) at concentrations from 2-2000 ng/ml. Data illustrated are duplicate determinations; repeated experiments yielded similar results.

Table 1. Comparison of the Fatty Acids from the Phospholipase D-Generated Phosphatidylethanol in Wild Type and Multidrug Resistant MCF-7 Cells[1]

Cell Line	Fatty Acid (relative per cent)						
	14:0	16:1	16:0	18:0	18:1	18:2	20:4
Wild Type MCF-7	10.8	7.8	36.7	23.6	23.5	1.8	1.4
	(1.4)	(0.8)	(4.1)	(0.5)	(3.2)	(0.3)	(0.1)
R300 MCF-7	6.0	4.7	35.0	23.0	25.7	1.6	1.2
	(2.1)	(0.6)	(2.6)	(3.3)	(4.4)	(0.4)	(0.3)

[1]Cells were incubated in the presence of TPA (200 ng/ml) for 20 min and the lipids were resolved, collected, and analyzed by gas chromatography-mass spectrometry as described in the Methods and Materials. The data represent the relative percent of the fatty acids determined by the integration of the reconstructed ion chromatograms. The values are the mean of triplicate determinations (standard error). There were no significant differences at $p < 0.05$; Students t-test. R300 MCF-7 = multidrug resistant MCF-7 cell line that is 300 fold resistant to adriamycin.

To verify the results from the studies that examined the changes in the cellular mass of PEt, experiments using cells radiolabeled with [^{3}H]myristic acid were conducted. This protocol has been used successfully in previous work with other cell lines to preferentially radiolabel a phospholipid substrate pool that serves as a target of PLD (5,6). Radiolabeling of the MCF-7 cells resulted in a predominant metabolism of [^{3}H]myristic acid into cellular PC with lesser amounts in the other phospholipids. The radiolabeling profiles of the phospholipids (relative % of total ^{3}H) were as follows (values listed as WT vs. R300) PC, 62 vs. 59; PE, 16 vs. 15; PS+PI, 13 vs. 16; SPM, 9 vs. 10. These data demonstrate that the radiolabeling patterns were similar in both cell types.

Subsequent studies examined both the time and concentration dependency of the PLD response. These experiments employed cells that were radiolabeled with [^{3}H]myristic acid and quantitated the production of radiolabeled PEt. In R300 cells, the production of ^{3}H-labeled PEt was maximal at approximately 200 ng TPA/ml, and in the R300 cells the ED_{50} for TPA-stimulated PEt production was ~7 ng TPA/ml (Figure 2). WT cells showed an ED_{50} for TPA-stimulated PEt production of ~30 ng TPA/ml, maximal production of [^{3}H]PEt at 200 ng TPA/ml, and a 4.2-fold lower TPA-stimulated production of PEt when compared to the R300 cells. Compared to the WT cells, the R300 cells generated approximately 3-5 times more TPA-stimulated [^{3}H]PEt at each of the time points examined (Figure 2). When exposed to TPA at 200 ng/ml and varying concentrations of ethanol (0.5, 1.0, and 2.0%), the expected concentration-dependent production of PEt was observed. At each of the concentrations tested, the R300 cells generated 3-6 fold more PEt than did the WT cells (data not shown). These findings verify the data obtained from the studies that determined the mass levels of PEt.

Since the physiological products resulting from PLD activity are PA and DAG, these metabolites were also assessed after exposure of WT and R300 cells to TPA. Figure 3 shows the

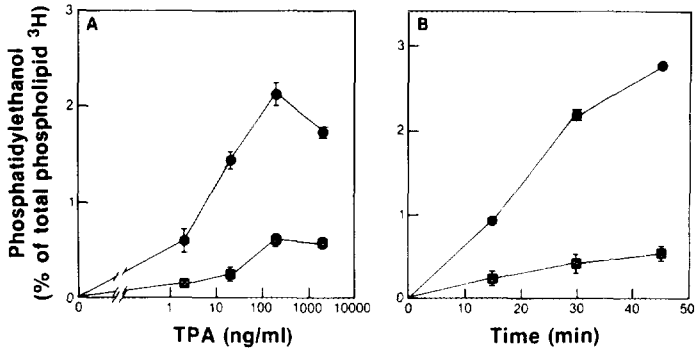

Figure 2. Time- and Concentration-dependency of Phorbol Ester-stimulated Phospholipase D Activity in Wild Type and Multidrug Resistant MCF-7 Cells: Analysis of Radiolabeled Phosphatidylethanol. Cell cultures of wild type (■) and multidrug resistant (●) were radiolabled with [^{3}H]myristic acid (0.5 μCi/ml; 24 hr) and then exposed to phorbol ester (TPA; increasing concentraions; panel A) for various time periods (panel B) in the presence of ethanol (2%). Data represent the mean of triplicate determinations; error bars represent standard deviations.

results of experiments using cells that were radiolabeled with [^{3}H]myristic acid and treated the cells with TPA. The time course for the production of radiolabeled PA and DAG was determined in the absence of ethanol. Basal levels of DAG were similar in both cell types (~1 mol % of PC). However when stimulated with TPA, the DAG levels in R300 cells reached 3.5 mol % of PC at the 10 min time point and were approximately 3.5x the levels observed in the WT cells. PA levels rose for at least 20 minutes in the R300 cells and reached a maximal level of approximately 2 mol % of PC that was 2-fold higher than PA levels found in WT cells.

A possible correlation between the TPA-stimulated PLD activity and the degree of resistance to adriamycin was examined. This comparison used several multidrug resistant MCF-7

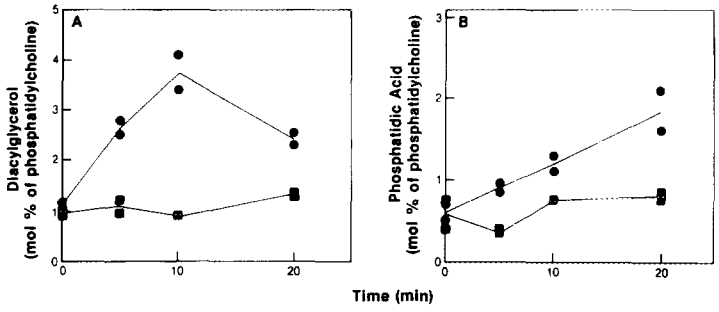

Figure 3. Time Course of Phorbol Ester-stimulated Production of Diacylglycerol and Phosphatidic Acid in Wild Type and Multidrug Resistant MCF-7 Cells. Cell cultures of wild type (■) and multidrug resistant cells (●) were exposed to phorbol ester (TPA; 200 ng/ml) without ethanol for various time periods. The cellular diacylglycerol and the phosphatidic acid were extracted, visualized, and the mass of the different analytes was quantitated after charring and subsequent photodensitometry. Data points illustrate duplicate determinations.

cells that express increasing levels of resistance to adriamycin and a concomitant elevated expression of P-gp, the multidrug transporter (14). The relative PLD activity was determined by monitoring the mass of PEt produced after exposure to TPA for 20 min. Comparisons using cell lines with varying degrees of resistance revealed a positive correlation between PLD activity and increasing drug resistance ($r = 0.817$) (Figure 4). Further examination of a MCF-7 cell line transfected with the MDR-1 cDNA for P-gp corroborated the previous findings (data not shown). These findings indicated the PLD activity increases with increasing capacity for drug resistance.

DISCUSSION This report is the first to present evidence demonstrating an increased activity of an agonist-stimulated PLD in cells expressing the multidrug resistance phenotype. Furthermore, in contrast to most previous studies of PLD that rely of radiolabeling techniques, this communication presents findings based on mass quantities of the cellular products of PLD. Studies using [^{3}H]myristic acid labeled cells corroborated the data obtained with the mass measurements, and demonstrated the time- and concentration dependent nature of the response. The finding that the majority of the ^{3}H radiolabeled the PC of both the WT and R300 cells suggests that PC can serve as a substrate for PLD in both cell types. The PEt species, as determined by the analysis of the constituent fatty acids, were similar in both the WT and R300 cells. This finding suggests the TPA-activated PLD hydrolyzes similar substrate pools in both cell types. Collectively, this evidence argues for a mechanism in which the level of agonist-activated PLD is elevated in the R300 cells, and that the increased PLD activity hydrolyzes more of a similar substrate pool.

The correlation between the increased PLD activity and increasing drug resistance suggests the P-gp activity and the phospholipase activity may be related. With respect to this putative relationship, it should be noted that the resistant cells demonstrate a constitutive ability to eliminate

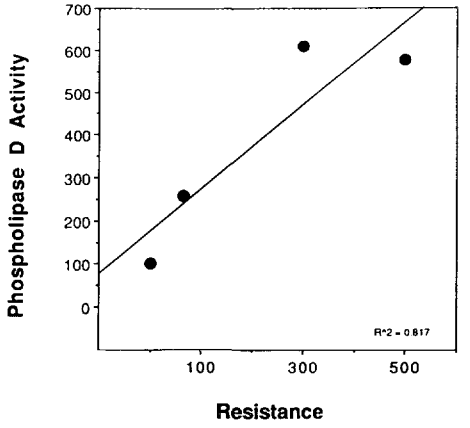

Figure 4. Correlation Between Phospholipase D Activity and Capacity for resistance to Adriamycin in Wild Type and Multidrug Resistant MCF-7 Cells. Phospholipase D activity was measured as described in the Legend to Figure 1. For the purposse of the illustration, the data is presented such that the relative phospholipase D activity in the wild type cells is set at a value of 100. Data points represent the means from 3-7 different determinations.

drugs. However, under resting conditions, the PLD activities are similar in the WT and the R300 cells (Figures 1 and 2). These data argue for a relationship in which the P-gp activity (or an unidentified cellular component) might promote or accelerate the coupling of the TPA-stimulus to the activation PLD in the R300 cells. That is, the elevated P-gp activity may optimize the membrane environment for signal-response coupling to the PLD activity.

A plausible alternative would include an increased protein kinase C (PKC) activity in the multidrug resistant MCF-7 cells. Such a correlation between PKC activity and P-gp expression in MCF-7 cells has been reported (23). Supporting this explanation are findings that show the R300 cells contain a higher level of PKC localized in the nucleus (24). Since PKC is a receptor for TPA, the differences in the apparent sensitivity to TPA (ED_{50} for R300 ~ 7 ng TPA/ml vs. ED_{50} for WT ~ 30 ng TPA/ml) also suggests a functional role for PKC in this system. The finding that the MCF-7 cell transfected with the MDR-1 gene for P-gp also shows an elevated level of the TPA-stimulated PLD activity supports the speculation that the PLD and the multidrug transporter activities are possibly linked in some manner that couples these activities to a common regulator. Definitive conclusions relative to the roles that PLD and/or PKC may play in the physiology of the multidrug resistance phenotype will require further investigation.

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