Phorbol ester selectively stimulates the phospholipase D-mediated hydrolysis of phosphatidylethanolamine in multidrug-resistant MCF-7 human breast carcinoma cells

Zoltan KISS,*† Masahiro TOMONO* and Wayne B. ANDERSON†

*The Hormel Institute, University of Minnesota, Austin, MN 55912, and †Laboratory of Cellular Oncology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

The phospholipase D (PLD)-mediated synthesis of phosphatidylethanol (PtdEtOH) and the hydrolysis of phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) were examined in drug-sensitive and multidrug-resistant lines of MCF-7 human breast carcinoma cells. In drug-sensitive (MCF-7/WT) cells, the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) failed to enhance either the synthesis of PtdEtOH or the hydrolysis of either phospholipid. In the drug-resistant (MCF-7/MDR) cells, 100 nM PMA greatly enhanced both the synthesis of PtdEtOH (~ 21-fold) and the hydrolysis of

PtdEtn (~ 29-fold), but had no effect on the hydrolysis of PtdCho. The PLD activators sphingosine and H₂O₂ were found to elicit only a slight (1.28–1.4-fold) stimulatory effect on PtdCho hydrolysis in both the MCF-7/WT and MCF-7/MDR cell types, and had only a small effect on PtdEtn hydrolysis in the MCF-7/WT cells as well. However, these agents significantly (~ 2.6–3.5-fold) stimulated PtdEtn hydrolysis in the MCF-7/MDR cells. These data indicate that MCF-7/MDR cells contain a PtdEtn-specific PLD activity which can be selectively stimulated by PMA, sphingosine and H₂O₂.

INTRODUCTION

An important question in clinical oncology is to understand the biochemical basis for the onset of resistance to anticancer drugs such as adriamycin and vinblastin, which are derived from natural products. Multidrug resistance (MDR) arises when cells selected for resistance to a single drug develop cross-resistance to other chemotherapeutic drugs [1,2]. A common feature of the MDR phenotype is the overexpression of a membrane-associated P-glycoprotein which appears to function as a drug-efflux pump to decrease the intracellular accumulation of drugs [3,4]. In addition to overexpression of P-glycoprotein, other mechanisms also have been implicated to modulate MDR, including changes in transmembrane signalling pathways. Protein kinase C (PKC) activity has been shown to be elevated in a number of drug-resistant cell types [5–8]. Other studies have shown that treatment of MDR cells with agents which alter PKC activity also altered the drug-resistant phenotype [5,6,9,10], further suggesting a role for PKC in multidrug resistance.

Since PKC is regulated by diacylglycerol generated in response to stimulation of various phospholipase activities, changes in phospholipase activities also might be expected in MDR cells. In addition, other observations also suggest that phospholipase activities may be changed in multidrug resistance. Physical changes in membrane structure, a consequence of altered phospholipid composition, have been linked to alterations in the sensitivity of cells to the cytotoxic effects of anticancer drugs [11–15]. Further, it has been suggested that phosphatidylethanolamine (PtdEtn) can activate the ATPase activity of the P-glycoprotein transporter [16]. These findings suggested that it would be of interest to extend our investigations of the biological and regulatory properties of phospholipases to drug-sensitive (MCF-7/WT) and multidrug-resistant (MCF-7/MDR) human breast carcinoma cells.

The hydrolysis of phospholipids by hormone- and phorbol ester-stimulated phospholipase D (PLD) results in the formation of phosphatidic acid [17,18]. Phosphatidic acid can serve as a messenger molecule with the potential to regulate cell growth [19–24], exocytosis [25–30], as well as other cellular processes (reviewed in [31]). In view of the emerging role of PLD in signal transduction, it is important to characterize the regulatory properties of PLD and to identify the phospholipid pools which can serve as substrates. There is agreement that activators of PKC, in addition to GTP, ATP and various growth-regulatory molecules, are prominent regulators of PLD [17,18,31]. With regard to substrate specificity, a number of laboratories have reported only the hydrolysis of phosphatidylcholine (PtdCho) by activated PLD, although in many cases this was the only phospholipid examined [17,18]. In a more recent study with microsomal PLD, Horwitz and Davis [32] also observed that PtdCho appeared to be the substrate of PLD, although they did note some hydrolysis of PtdEtn.

In contrast, we have noted that activation of PLD in NIH 3T3 fibroblasts by the PKC activator phorbol 12-myristate 13-acetate (PMA) [33,34], ATP [35], GTP [35], sphingosine [35,36], staurosporine [36], H₂O₂ [37] or hormones [38] led to comparable hydrolysis of both PtdCho and PtdEtn. Recently, stimulation of PtdEtn hydrolysis in response to activators of PLD also has been reported in several other cell types [39–46]. These observations raise the possibility that increased PtdEtn hydrolysis may occur only in a limited number of cell types and that it may have physiological function(s) different from that of PtdCho hydrolysis. If so, it might be expected that, under certain physiological or pathological conditions, only the PtdEtn-hydrolysing system may be modified.

In this paper, we have assessed changes in the hydrolysis of PtdCho and PtdEtn by PLD in MCF-7/WT and MCF-7/MDR cells treated with the PLD activators PMA, sphingosine and

Abbreviations used: PLD, phospholipase D; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; WT, drug-sensitive; MDR, multidrug-resistance.

† To whom correspondence should be addressed.

H₂O₂. Treatment of drug-sensitive MCF-7/WT cells with PMA had little effect on the hydrolysis of either PtdCho or PtdEtn. However, PMA treatment of MCF-7/MDR cells had a pronounced stimulatory effect on the PLD-mediated hydrolysis of PtdEtn, but not on the hydrolysis of PtdCho. These results indicate that the PLD-mediated hydrolysis of PtdEtn is differentially and selectively regulated by PKC activators in MCF-7/MDR cells.

MATERIALS AND METHODS

Materials

PMA, sphingosine, H₂O₂ and Dowex-50 (H⁺ form) were purchased from Sigma; [methyl-¹⁴C]choline chloride (50 mCi/mmol), [2-¹⁴C]ethanolamine (50 mCi/mmol), L-[U-¹⁴C]serine (150 mCi/mmol), myo-[2-³H]inositol (15 mCi/mmol) and [1-¹⁴C]palmitic acid (60 mCi/mmol) were from Amersham; phosphatidylethanol (PtdEtOH) was from Avanti Polar Lipids, and tissue-culture reagents were purchased from GIBCO.

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, U.S.A.). MCF-7 cells were grown in Richter's Iscove's modified Eagle medium (IMEM) supplemented with 2 mM glutamine, 12 mg/ml L-proline, 50 µg/ml gentamicin and 10% fetal bovine serum. Cells were passaged twice weekly and monitored routinely for their resistance to adriamycin. The MCF-7/MDR cells were periodically reselected by growing them in the presence of increasing concentrations of adriamycin (up to 10 µM) for a period of approx. 2 months.

Measurement of formation of PtdEtOH in MCF-7 cells

MCF-7 cells were grown in 6-well tissue-culture dishes for 48 h to about 80% confluency, and labelled with [1-¹⁴C]palmitic acid (0.35 mCi/ml) for the last 24 h. Cells were washed, incubated in fresh IMEM for an additional 2 h (to minimize the amount of unesterified labelled palmitic acid), and then treated with PMA in the presence of 200 mM ethanol for 30 min. Incubations were performed in an incubator at 37°C and 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.).

Measurement of PLD-mediated hydrolysis of PtdCho and PtdEtn in MCF-7 cells

MCF-7 cells were grown in 6-well tissue-culture dishes for 48 h (up to 80% confluency) in the presence of [¹⁴C]choline (0.6 mCi/ml) or [¹⁴C]ethanolamine (0.4 mCi/ml). Cells were washed and incubated in fresh IMEM for 3 h, followed by treatments with PMA as described in Figure 2 in the presence of 20 mM choline or 2 mM ethanolamine, as appropriate. Incubations were terminated as indicated above for the [¹⁴C]palmitate-labelled cells. Fractionation of choline and ethanolamine metabolites was performed on Dowex-50W (H⁺)-packed columns (Bio-Rad Econo columns; 1 ml bed volume) with minor modifica-

tions of the procedure described by Cook and Wakelam [47]. The initial flow-through (4.5 ml), along with a following 3.5 ml or 5 ml water wash, contained glycerophosphoethanolamine or glycerophosphocholine, respectively. Ethanolamine phosphate and choline phosphate were then eluted by 15 ml and 20 ml of water, respectively. Finally, ethanolamine and choline were eluted by 12 ml and 20 ml of 1 M HCl, respectively. The metabolites of [¹⁴C]ethanolamine and [¹⁴C]choline were further identified by t.l.c. [33]. Phospholipids were separated as described previously [48].

In some experiments, cells were grown in 150 mm-diameter dishes in the presence of either [methyl-¹⁴C]choline (0.3 mCi/ml) or [2-¹⁴C]ethanolamine (0.25 mCi/ml) for 48 h. Cells were washed, incubated in fresh IMEM for 3 h, and harvested by scraping from two to four dishes. At this step, about 95% of cells were viable, determined by the Trypan Blue dye-exclusion assay. Scraped cells were incubated in the presence of 20 mM choline or 2 mM ethanolamine for 20 min to produce large intracellular pools of unlabelled bases and their phosphorylated intermediates (this serves to prevent metabolism of newly formed ¹⁴C-labelled compound during the subsequent incubation period; see [38] and [49]). Scraped cells were collected by centrifugation (10 min at 500 g), and portions (0.2 ml) of the cell suspensions [(1.2–1.4) × 10⁶ cells/ml] were treated with 100 nM PMA (final volume 0.25 ml) in the presence of unlabelled choline (20 mM) or ethanolamine (2 mM). The background levels of [¹⁴C]choline and [¹⁴C]ethanolamine in suspended cells were 60–80% lower than in attached cells. Incubations were terminated by addition of 4 ml of chloroform/methanol (1:1, by vol.).

RESULTS

Comparison of effects of PMA on PtdEtOH synthesis in MCF-7/WT and MCF-7/MDR cells

PLD is uniquely capable of synthesizing phosphatidylalcohol from phospholipid acceptors and alcohol [17,18]. Since production of phosphatidylalcohol appears to be a reliable measure of PLD activity, we determined the effect of PMA on PtdEtOH formation in the drug-sensitive and drug-resistant MCF-7 cell lines. In the presence of serum (but not in its absence), MCF-7/WT cells synthesized a small amount of PtdEtOH, which was somewhat inhibited, rather than enhanced, by increasing concentrations of PMA (Figure 1). In contrast, in MCF-7/MDR cells PMA greatly enhanced PtdEtOH formation; near half-maximal (~9-fold) and maximal (~21-fold) stimulatory effects were observed with 10 and 100 nM concentrations of PMA respectively (Figure 1).

Comparison of the effects of PMA on the hydrolysis of PtdCho and PtdEtn in MCF-7/WT and MCF-7/MDR cells

Although PtdEtOH is specifically synthesized by PLD, this reaction does not provide information about the nature of physiological phospholipid substrates. To examine specifically the substrate role of PtdCho and PtdEtn, we used cells in which each of these phospholipids was prelabelled in the choline or ethanolamine moiety, respectively. Special care was taken to perform the treatments in the presence of 20 mM choline or 2 mM ethanolamine, as appropriate, to prevent (or at least to decrease greatly) further metabolism of phospholipid-derived [¹⁴C]choline or [¹⁴C]ethanolamine [33,38,49]. As shown in Figure 2(a), [¹⁴C]choline-labelled MCF-7/WT cells produced more [¹⁴C]choline from [¹⁴C]PtdCho than did [¹⁴C]choline-labelled MCF-7/MDR cells. Importantly, PMA failed to enhance significantly the formation of [¹⁴C]choline in either cell line; in fact, in

Figure 1 Effects of PMA on the formation of PtdEtOH in [^{14}C]palmitate-labelled drug-sensitive and drug-resistant MCF-7 cells

Attached MCF-7/WT (●) and MCF-7/MDR (▲) cells, prelabelled with [^{14}C]palmitic acid, were treated for 30 min with increasing concentrations (10–100 nM) of PMA in the presence of 200 mM ethanol. The [^{14}C] content of PtdCho and PtdEtn in the two cell lines was in the range of 555 000–612 000 and 162 000–189 000 d.p.m./ 10^6 cells respectively. Data are means $\pm$ S.E.M. of three independent incubations. This experiment was repeated twice with similar results.

Figure 2 Effects of PMA on the hydrolysis of PtdCho and PtdEtn in attached drug-sensitive and drug-resistant MCF-7 cells

Attached MCF-7/WT (●) and MCF-7/MDR (▲) cells, prelabelled with [^{14}C]choline (a) or [^{14}C]ethanolamine (b), were treated for 30 min with increasing concentrations (0–100 nM) of PMA. The [^{14}C] content of PtdCho and PtdEtn in the two cell lines was in the range of 780 000–990 000 and 710 000–860 000 d.p.m./ 10^6 cells respectively. Each point represents the means $\pm$ S.E.M. of three independent incubations. Similar results were obtained in another experiment (performed in triplicate).

MCF-7/WT cells PMA treatment inhibited PtdCho degradation (Figure 2a). The formation of [^{14}C]ethanolamine from [^{14}C]PtdEtn also was more pronounced in MCF-7/WT cells than in MCF-7/MDR cells (Figure 2b). However, although PMA treatment of labelled MCF-7/WT cells had no effect on

Figure 3 Effects of PMA on the hydrolysis of PtdCho and PtdEtn in drug-sensitive and drug-resistant MCF-7 cells in suspension

Suspended MCF-7/WT (●) and MCF-7/MDR (▲) cells, prelabelled with [^{14}C]choline (a) or [^{14}C]ethanolamine (b), were treated for 60 min with increasing concentrations (0–100 nM) of PMA. Each point represents the mean $\pm$ S.E.M. of three independent incubations. Similar results were obtained in two other experiments each performed in triplicate.

[^{14}C]ethanolamine formation, PMA significantly stimulated the hydrolysis of [^{14}C]PtdEtn in labelled MCF-7/MDR cells (Figure 2b).

Since in certain cellular systems the cellular phospholipids phosphatidylserine and phosphatidylinositol also may serve a limited role as substrates (reviewed in [32]), we also examined the effects of PMA on the hydrolysis of these phospholipids in the MCF-7/MDR cells prelabelled with either [^{14}C]serine or [^3H]inositol. Although release of both [^{14}C]serine and [^3H]inositol from the respective labelled phospholipids was detectable, formation of these products was not enhanced by 100 nM PMA (results not shown). Thus it seems unlikely that these phospholipids contribute significantly to the PMA-induced formation of PtdEtOH observed in MCF-7/MDR cells (Figure 1).

In the drug-sensitive MCF-7/WT cells, the formation of [^{14}C]choline from [^{14}C]PtdCho was pronounced, but was inhibited by PMA. In principle, it was possible that stimulation of PtdCho hydrolysis by a serum factor masked a stimulatory effect of PMA. During the course of these experiments we noted that in suspended cells, in contrast with attached cells, serum had no significant stimulatory effect on PtdCho hydrolysis. Thus we next examined the effects of PMA on phospholipid hydrolysis in suspended MCF-7 cells. As expected, PMA had no inhibitory effect on [^{14}C]choline formation in [^{14}C]choline-labelled MCF-7/WT cells in suspension (Figure 3a). Further, PMA was also observed to have no effect on PtdCho hydrolysis in drug-resistant MCF-7/MDR cells in suspension even during an extended (60 min) incubation period (Figure 3a). When PMA was added to [^{14}C]ethanolamine-labelled MCF-7 cells in suspension, it failed to elicit an increase in [^{14}C]PtdEtn hydrolysis in the drug-sensitive cells (Figure 3b). However, PMA again was observed to enhance significantly [^{14}C]ethanolamine formation in the MDR line (Figure 3b). Thus the effects of PMA on phospholipid hydrolysis in suspended cells were found to be qualitatively similar to those observed in attached cells; i.e., PMA stimulated only PtdEtn hydrolysis and only in the MCF-7/MDR cells.

Table 1 Comparison of the effects of sphingosine, H₂O₂ and PMA on the hydrolysis of PtdCho and PtdEtn in drug-sensitive and drug-resistant MCF-7 cells

Attached cells, prelabelled with [¹⁴C]choline or [¹⁴C]ethanolamine, as indicated, were treated with sphingosine (30 µM), H₂O₂ (2 mM) and/or PMA (100 nM) for 20 min. Data are means ± S.E.M. of four independent incubations. This experiment was repeated twice with similar results.

Addition	Formation of (d.p.m./20 min per 10 ⁶ d.p.m. of [¹⁴ C]phospholipid):			
	[¹⁴ C]Choline		[¹⁴ C]Ethanolamine	
	MCF-7/WT	MCF-7/MDR	MCF-7/WT	MCF-7/MDR
None	7550 ± 560	4380 ± 370	16450 ± 530	5090 ± 510
H ₂ O ₂	11830 ± 440	5390 ± 820	21250 ± 760	13040 ± 820
Sphingosine	10560 ± 1890	6110 ± 310	21130 ± 1620	17790 ± 450
PMA	8030 ± 810	4580 ± 220	20720 ± 3170	22780 ± 960
Sphingosine + PMA	10160 ± 1090	4460 ± 390	23100 ± 3630	46470 ± 1510

Comparison of the effects of sphingosine, H₂O₂ and PMA on the hydrolysis of PtdCho and PtdEtn in MCF-7/WT and MCF-7/MDR cells

To characterize further possible differences in the phospholipase activities present in drug-sensitive and drug-resistant MCF-7 cells, we next determined the effects of other PLD activators, such as sphingosine [35,36,50] and H₂O₂ [46], which appear to stimulate phospholipid hydrolysis through PKC-independent mechanisms. Each compound was used at a maximally effective concentration for an optimal period of incubation (20 min), as determined in preliminary experiments. As shown in Table 1, sphingosine and H₂O₂ stimulated the hydrolysis of both PtdCho and PtdEtn hydrolysis in both the drug-sensitive and drug-resistant MCF-7 cell types. However, the stimulatory effects of these compounds on PtdCho hydrolysis in the MCF-7/WT and MCF-7/MDR cells, as well as on PtdEtn hydrolysis in the MCF-7/WT line, were relatively (1.28–1.4-fold) small. In contrast, treatment of MCF-7/MDR cells with H₂O₂ and sphingosine stimulated PtdEtn hydrolysis by 2.56- and 3.5-fold, respectively (Table 1). In agreement with data in Figures 2 and 3, PMA enhanced only PtdEtn hydrolysis, and only in the MCF-7/MDR line (Table 1). Previously, we reported that in NIH 3T3 fibroblasts PMA and sphingosine additively or synergistically (depending on the length of incubation) enhanced PtdEtn hydrolysis [36]. As presented in Table 1, in the drug-resistant cells PMA and sphingosine stimulated PtdEtn hydrolysis about 4.5- and 3.5-fold, respectively, whereas in combination they had a 9.1-fold stimulatory effect. In comparison, in the drug-sensitive cells PtdEtn hydrolysis was stimulated only 1.4-fold by PMA plus sphingosine (Table 1).

Formation of glycerophosphocholine and glycerophosphoethanolamine in MCF-7/WT cells

A somewhat controversial finding in these studies was that MCF-7/WT cells produced relatively large amounts of choline and ethanolamine from the respective labelled phospholipids (Figures 2 and 3), even though the PLD activity in these cells appeared to be relatively low (Figure 1). Therefore, it was considered that a significant portion of the production of choline and ethanolamine in the unstimulated cells could involve non-PLD-mediated mechanisms. If so, a possible mechanism might be that degradation of phospholipids by type A phospholipases would occur at higher rates in the drug-sensitive than in the drug-resistant cells. Choline and ethanolamine could then be formed from glycerophosphocholine and glycerophosphoethanolamine

produced by the respective phosphodiesterases. We examined this possibility, and found that in ¹⁴C-labelled MCF-7/WT cells the formation of [¹⁴C]glycerophosphocholine (21 200 d.p.m./2 h per 10⁶ d.p.m. of [¹⁴C]PtdCho) and [¹⁴C]glycerophosphoethanolamine (53 600 d.p.m./2 h per 10⁶ d.p.m. of PtdEtn) from the respective ¹⁴C-labelled phospholipids occurred at rates 3–5 times those found in the MCF-7/MDR cells. Similarly, untreated attached (Figure 2, Table 1) or suspended (Figure 3) MCF-7/WT cells produced more [¹⁴C]choline and [¹⁴C]ethanolamine than did MCF-7/MDR cells. In addition, when ¹⁴C-labelled MCF-7/WT cells were collected by scraping in a medium containing 20 mM glycerophosphocholine or glycerophosphoethanolamine (i.e., cells were scrape-loaded with these compounds), we observed about 50–60% decrease in the formation of [¹⁴C]choline and [¹⁴C]ethanolamine during a subsequent 60 min incubation period. These data indicate that in unstimulated MCF-7/WT cells a major portion of both choline and ethanolamine should be derived by the sequential actions of type A phospholipases and the corresponding phosphodiesterases. It should be added that there was some variability in the formation of [¹⁴C]ethanolamine in the attached untreated MCF-7/MDR cells. Thus, although in the experiment shown in Figure 2(b) drug-sensitive cells produced about 50 times more [¹⁴C]ethanolamine than the drug-resistant cells, in the experiment shown in Table 1, the former cell line produced only 3 times more [¹⁴C]ethanolamine. This variability is partly due to the different background levels of the precursor [¹⁴C]glycerophosphoethanolamine in the various experiments (results not shown). Further characterization of the apparently higher activities of type A phospholipases in the MCF-7/WT cells and the conversion of glycerophosphoethanolamine into ethanolamine is beyond the scope of the present investigation. When examined in suspended MCF-7/WT cells, PMA, sphingosine and H₂O₂ were found not to modify the formation of [¹⁴C]glycerophosphocholine and [¹⁴C]glycerophosphoethanolamine from the respective [¹⁴C]phospholipid (results not shown).

DISCUSSION

The results presented demonstrate that PMA treatment of drug-resistant MCF-7/MDR cells selectively stimulates the PLD-mediated hydrolysis of PtdEtn, whereas similar treatment of drug-sensitive MCF-7/WT cells has little effect on PLD activity. This is the first clear example of a cellular system where PMA specifically enhances the hydrolysis of PtdEtn, while having little effect on the hydrolysis of PtdCho. This appears to indicate, in agreement with the data of Wang et al. [51], that PtdEtn and

PtdCho may be hydrolysed by different PLD activities. Thus, it might be expected that the relative levels of these PLD activities might differ in various cell types. Based on the measured rate of PtdCho and PtdEtn hydrolysis, a number of cell types examined appear to possess only the PtdCho-specific PLD activity (reviewed in [17,18]). On the other hand, NIH 3T3 fibroblasts [33–38], endothelial cells [46], rat mesangial cells [41–44], human amnion cells [45], rat mammary cells [40], HeLa cells [39], baby-hamster kidney cells [33] and HL60 human leukaemic cells [33] clearly express both PtdCho- and PtdEtn-hydrolysing PLD activities.

Although PMA failed to stimulate PtdCho hydrolysis in these MCF-7 lines, a low level of PtdCho-specific PLD activity appears to be present both in the drug-sensitive and drug-resistant cells, based on the low levels of stimulation noted with H_2O_2 and sphingosine. The reason why this residual PtdCho-PLD activity was not enhanced by PMA is not clear. One possibility is that mammalian cells may contain several isoforms of PtdCho-PLD, each of which exhibiting different regulatory properties. The form of PtdCho-hydrolysing PLD present in MCF-7 cells may respond to sphingosine and H_2O_2 , but not to PKC activators such as PMA. Another possibility is that the effect of PMA on PtdCho-specific PLD, unlike that of sphingosine and H_2O_2 , may require an intermediate step which is impaired in the MCF-7 cells. Alternatively, the levels of a PKC isoenzyme or a possible intermediate PKC substrate, which may serve to mediate the effects of PMA on the hydrolysis of PtdEtn and PtdCho, could be altered in these cells. Previous studies have established that the PKC isoenzyme pattern is altered in these cells, with the PKC- α isoform being markedly elevated in MCF-7/MDR cells [52]. In either case, the observations that PMA, sphingosine and H_2O_2 each had significant stimulatory effects only on PtdEtn hydrolysis, and only in MCF-7 cells exhibiting the drug-resistant phenotype, suggests that stimulation of PtdEtn hydrolysis by these agents involves a common PLD enzyme which is different from the PtdCho-hydrolysing PLD activity.

The finding that the PtdEtn-hydrolysing system is independently regulated by the PKC system also suggests that PtdEtn hydrolysis may have a physiological function different from that of PtdCho hydrolysis. Since increased PtdEtn hydrolysis specifically occurs in the drug-resistant cells, it is possible that increased PtdEtn hydrolysis contributes, together with other factors, to the modulation of increased drug efflux and/or decreased drug influx. In light of the known fusogenic property of phosphatidic acid, it is possible that the PLD-mediated formation of this phospholipid from PtdEtn might stimulate drug efflux by facilitating the interaction between drug-accumulating intramembrane vesicles and plasma membrane [53–56]. Another possibility is that alterations in the plasma-membrane levels of PtdEtn and phosphatidic acid result in alterations in membrane structure which influence the activity of the transmembrane P-glycoprotein transporters. In this regard, PtdEtn recently has been reported to activate P-glycoprotein ATPase activity [16]. If there is indeed a correlation between MDR and PMA-sensitive PtdEtn-specific PLD, then a similar pattern of PLD activation should be observed in any independently cloned resistant lines of breast carcinoma cells. This latter possibility is at present under investigation in our laboratory.

We are grateful to Mrs. K. S. Crilly for technical assistance and to Mrs. C. Perleberg for secretarial assistance. This work was supported by the Hormel Foundation.

REFERENCES

- 1 Riordan, J. R. and Ling, V. (1985) *Pharmacol. Ther.* **28**, 51–75
- 2 Pastan, I. and Gottesman, M. M. (1987) *N. Engl. J. Med.* **316**, 1388–1393
- 3 Gottesman, M. M. and Pastan, I. (1988) *J. Biol. Chem.* **263**, 12163–12166
- 4 Juranka, P. F., Zastawny, R. L. and Ling, V. (1989) *FASEB J.* **3**, 2583–2592
- 5 Fine, R. L., Patel, J. and Chabner, B. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 582–586
- 6 O'Brian, C. A., Fan, D., Ward, N. E., Seid, C. and Fidler, I. J. (1989) *FEBS Lett.* **246**, 78–82
- 7 Aguino, A., Warren, B. S., Omichinski, J., Hartman, K. D. and Glazer, R. (1990) *Biochem. Biophys. Res. Commun.* **166**, 723–728
- 8 Lee, S. A., Karaszewicz, J. W. and Anderson, W. B. (1992) *Cancer Res.* **52**, 3750–3759
- 9 Ferguson, P. J. and Cheng, Y. C. (1987) *Cancer Res.* **47**, 433–441
- 10 Dong, Z., Ward, N. E., Fan, D., Gupta, K. P. and O'Brian, C. A. (1991) *Mol. Pharmacol.* **39**, 563–569
- 11 Glaubiger, A. R., Magrath, I. T. and Joshi, A. (1983) *Cancer Res.* **43**, 5533–5537
- 12 Siegfried, J. A., Kennedy, K. A., Sartorelli, A. C. and Tritton, T. R. (1983) *J. Biol. Chem.* **258**, 339–343
- 13 Montaudon, D., Vignand, P., Lodos-Gagliardi, D. and Robert, J. (1986) *Cancer Res.* **46**, 5602–5605
- 14 Escribe, P. V., Ferrer-Montiel, A. V., Ferragut, J. A. and Gonzalez-Kos, J. M. (1990) *Biochemistry* **29**, 7275–7282
- 15 Sinicropo, F. A., Dudeja, P. K., Bissonnette, B. M., Safa, A. R. and Brasitus, T. A. (1992) *J. Biol. Chem.* **267**, 24995–25002
- 16 Doige, C. A., Yu, X. and Sharom, F. J. (1993) *Biochim. Biophys. Acta* **1146**, 65–72
- 17 Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1–4
- 18 Billah, M. M. and Anthes, J. C. (1990) *Biochem. J.* **269**, 281–291
- 19 Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Biermann, A. J. and De Laat, S. W. (1986) *Nature (London)* **323**, 171–173
- 20 Yu, C. L., Tsai, M. H. and Stacey, D. W. (1988) *Cell* **52**, 63–71
- 21 Van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T. and Moolenaar, W. H. (1989) *Cell* **59**, 45–54
- 22 Knauss, T. C., Jaffer, F. E. and Abboud, H. E. (1990) *J. Biol. Chem.* **265**, 14457–14463
- 23 Van Corven, E. J., Van Rijswijk, A., Jalink, K., Van Der Bend, R. L., Van Blitterswijk, W. J. and Moolenaar, W. H. (1992) *Biochem. J.* **281**, 163–169
- 24 Fukami, K. and Takenawa, T. (1992) *J. Biol. Chem.* **267**, 10988–10993
- 25 Kinsky, S. C., Loader, J. E. and Benedict, S. H. (1989) *Biochem. Biophys. Res. Commun.* **162**, 788–793
- 26 Liscovitch, M. and Amsterdam, A. (1989) *J. Biol. Chem.* **264**, 11762–11767
- 27 Dunlop, M. and Metz, S. A. (1989) *Biochem. Biophys. Res. Commun.* **163**, 922–928
- 28 Stutchfield, J. and Cockcroft, S. (1993) *Biochem. J.* **293**, 649–655
- 29 Zhou, H. L., Fletcher, M. C., Foley, J. J., Saran, H. M., Tzimas, M. N., Winkler, J. D. and Torphy, T. J. (1993) *Biochem. Pharmacol.* **46**, 139–148
- 30 Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. C. (1993) *Cell* **75**, 1137–1144
- 31 Kiss, Z. (1990) *Prog. Lipid Res.* **29**, 141–166
- 32 Horwitz, J. and Davis, L. L. (1993) *Biochem. J.* **295**, 793–798
- 33 Kiss, Z. and Anderson, W. B. (1989) *J. Biol. Chem.* **264**, 1483–1487
- 34 Kiss, Z., Rapp, U. R., Pettit, G. R. and Anderson, W. B. (1991) *Biochem. J.* **276**, 505–509
- 35 Kiss, Z. and Anderson, W. B. (1990) *J. Biol. Chem.* **265**, 7345–7350
- 36 Kiss, Z. and Deli, E. (1992) *Biochem. J.* **288**, 853–858
- 37 Kiss, Z. (1994) *Biochem. J.* **298**, 93–98
- 38 Kiss, Z. (1992) *Biochem. J.* **285**, 229–233
- 39 Hii, C. S. T., Edwards, Y. S. and Murray, A. W. (1991) *J. Biol. Chem.* **266**, 20238–20243
- 40 Fisk, H. A. and Kano-Sueka, T. (1992) *J. Cell. Physiol.* **153**, 589–595
- 41 Kester, M., Simonson, M. S., McDermott, R. G., Baldi, E. and Dunn, M. J. (1992) *J. Cell. Physiol.* **150**, 578–585
- 42 Harris, W. E. and Bursten, S. L. (1992) *Biochem. J.* **281**, 675–682
- 43 Kester, M., Simonson, M. S., Mené, P. and Sedor, J. R. (1989) *J. Clin. Invest.* **83**, 718–723
- 44 Kester, M. (1993) *J. Cell. Physiol.* **156**, 317–325
- 45 Mizunuma, M., Tanaka, S., Kudo, R. and Kanoh, H. (1993) *Biochim. Biophys. Acta* **1168**, 213–219
- 46 Natarajan, V., Taher, M. M., Roehm, B., Parinandi, N. L., Schmid, H. H. O., Kiss, Z. and Garcia, J. G. N. (1993) *J. Biol. Chem.* **268**, 930–937
- 47 Cook, S. J. and Wakelam, M. J. O. (1989) *Biochem. J.* **263**, 581–587
- 48 Kiss, Z. (1976) *Eur. J. Biochem.* **67**, 557–561
- 49 Kiss, Z. (1992) *Eur. J. Biochem.* **209**, 467–473
- 50 Lavie, Y. and Liscovitch, M. (1990) *J. Biol. Chem.* **265**, 3868–3872
- 51 Wang, P., Anthes, J. C., Siegel, M. I., Egan, R. W. and Billah, M. M. (1991) *J. Biol. Chem.* **266**, 14877–14880
- 52 Blobe, G. C., Sachs, C. W., Khan, W. A., Fabbro, D., Stabel, S., Wetsel, W. C., Obeid, L. M., Fine, R. L. and Hannun, Y. A. (1993) *J. Biol. Chem.* **268**, 658–664

- 53 Wright, L. C., Dyne, M., Holmes, K. T. and Mountford, C. E. (1985) *Biochem. Biophys. Res. Commun.* **133**, 533–545
- 54 Arsenault, A. L., Ling, V. and Kartner, N. (1988) *Biochim. Biophys. Acta* **938**, 318–321
- 55 Garcia-Segura, L. M., Ferragut, J. A., Ferrer-Montiel, A. V., Escribe, P. V. and Gonzalez-Ros, J. M. (1990) *Biochim. Biophys. Acta* **1029**, 191–195
- 56 Garcia-Segura, L. M., Soto, F., Planells-Cases, R., Gonzalez-Kos, J. M. and Ferragut, J. A. (1992) *FEBS Lett.* **314**, 404–408

Received 11 February 1994/5 April 1994; accepted 20 April 1994