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Phorbol esters induce multidrug resistance in human breast cancer cells

(protein kinase C/drug efflux/drug resistance)

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ABSTRACT **Mechanisms responsible for broad-based resistance to antitumor drugs derived from natural products (multidrug resistance) are incompletely understood. Agents known to reverse the multidrug-resistant phenotype (verapamil and trifluoperazine) can also inhibit the activity of protein kinase C. When we assayed human breast cancer cell lines for protein kinase C activity, we found that enzyme activity was 7-fold higher in the multidrug-resistant cancer cells compared with the control, sensitive parent cells. Exposure of drug-sensitive cells to the phorbol ester phorbol 12,13-dibutyrate [P(BtO)$_2$] led to an increase in protein kinase C activity and induced a drug-resistance phenotype, whereas exposure of drug-resistant cells to P(BtO)$_2$ further increased drug resistance. In sensitive cells, this increased resistance was accompanied by a 3.5-fold increased phosphorylation of a 20-kDa particulate protein and a 35–40% decreased intracellular accumulation of doxorubicin and vincristine. P(BtO)$_2$ induced resistance to agents involved in the multidrug-resistant phenotype (doxorubicin and vincristine) but did not affect sensitivity to an unrelated alkylating agent (melphalan). The increased resistance was partially or fully reversible by the calcium channel blocker verapamil and by the calmodulin-antagonist trifluoperazine. These data suggest that stimulation of protein kinase C plays a role in the drug-transport changes in multidrug-resistant cells. This may occur through modulation of an efflux pump by protein phosphorylation.**

Cancer cells commonly manifest resistance to antineoplastic drugs during the course of treatment. A particular phenotype of resistant cells, called multidrug resistance (MDR), has been recognized and encompasses a broad pattern of resistance to anticancer drugs derived from natural products. Drugs affected include the anthracyclines, vinca alkaloids, colchicine, epipodophyllotoxins, actinomycin D, and other antibiotics. A common feature of MDR cells is a net decreased intracellular accumulation of drug, a finding ascribed to an increased efflux pump mechanism (1) and associated with the presence of a distinct membrane glycoprotein, the p170 glycoprotein.

Features of the MDR phenotype suggest a role for calcium-dependent biochemical events. MDR can be partially, or fully, reversed by calcium channel blockers such as verapamil and by calmodulin inhibitors such as trifluoperazine (F_3Pz) (2–4). Calmodulin inhibitors also inhibit Ca^{2+}-dependent protein kinases such as calmodulin kinase and protein kinase C (PKC) (5). When *in vitro* and intact cell protein phosphorylation profiles were examined in human breast cancer (6) and human small-cell lung cancer lines (7), we found a general increase in protein phosphorylation, as well as a specific increased phosphorylation of the 20-kDa region in lines with MDR. Lines sensitive to doxorubicin (adriamycin) or vincristine or that exhibited antimetabolite resistance did not have this increased 20-kDa phosphoprotein. Thus, to further define the role of PKC in MDR and to determine whether MDR could be induced by phorbol esters, we assessed changes in PKC activity, drug sensitivity, phosphorylation of the 20-kDa protein, and drug accumulation after exposure of cells to phorbol esters. We found that PKC activation was associated with increased drug resistance, increased phosphorylation of the 20-kDa region, and decreased intracellular accumulation of anticancer drugs. These changes were reversed by calcium channel blockers and calmodulin antagonists, findings that suggest a role for PKC in MDR in human cancer cells.

MATERIALS AND METHODS

Chemicals and Reagents. Doxorubicin, vincristine, and melphalan (L-phenylalanine mustard) were obtained from the Drug Synthesis Branch, National Cancer Institute (Bethesda, MD). Verapamil, F_3Pz, leupeptin, histone III$_S$, phosphatidylserine, diolene, phorbol 12,13-dibutyrate P(BtO)$_2$ and phorbol 13,20-diacetate P(AcO)$_2$ were obtained from Sigma. [^{14}C]Doxorubicin hydrochloride (17 mCi/mmol; 1 Ci = 37 GBq) and [^{14}C]melphalan (13 mCi/mmol) were from Stanford Research (Menlo Park, CA), [^{3}H]vincristine (8 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA), and [^{32}P]-orthophosphate was obtained from New England Nuclear (Boston, MA).

Cell Lines. The drug-sensitive wild-type parent line (MCF7 WT) was derived from human breast cancer cell line MCF7 stock cells. A subline (MCF7 Dox^R) was selected by serial passage in the presence of increasing concentrations of doxorubicin. These cell lines were supplied by K. H. Cowan (National Cancer Institute). MCF7 Dox^R was selected after 1 yr of drug exposure and was capable of growing in 10 μM doxorubicin (8). Both parental and mutant lines were passaged in improved minimal essential medium (IMEM; GIBCO) with 10% heat-inactivated fetal calf serum and were split weekly to maintain logarithmic growth. MCF7 Dox^R was passaged in drug-free media for at least eight passages before testing.

PKC Activity Assay. Harvested cells were disrupted by sonication in buffer A containing 20 mM Tris (pH 7.4), 2 mM EGTA, 5 mM 2-mercaptoethanol, 125 μg of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride. Particulate and soluble fractions at 4°C were separated by a 100,000 × *g* spin for 60 min in 2 ml of buffer A containing 0.1% Triton X-100. The particulate fraction was solubilized in buffer A with 1%

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Abbreviations: P(BtO)$_2$, phorbol 12,13-dibutyrate; MDR, multidrug resistance; PKC, protein kinase C; P(AcO)$_2$, phorbol 13,20-diacetate; WT, wild-type; Dox^R, doxorubicin (Adriamycin) resistant; IMEM, improved minimal essential medium; F_3Pz, trifluoperazine; PBS, phosphate-buffered saline.

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Triton X-100 for 15 min at 4°C. The total sample was then spun at 100,000 × *g* for 60 min, and the soluble fraction was collected. The samples were placed on DEAE-52 columns, and aliquots were eluted off with 90 mM NaCl solution by a previously described method (9). Aliquots (25 μg) of the homogenate, the soluble and the particulate fractions of MCF7 WT and MCF7 Dox^R cells were assayed for PKC activity with histone III$_S$ (1 mg/ml) as substrate. Assays were conducted in the presence of 50 mM Tris·HCl (pH 7.4) and 10 mM $MgCl_2$ alone or with phosphatidylserine (12 μg/ml), diolene (0.8 μg/ml) (diacylglycerol analogue), and 200 μM free $CaCl_2$ for measurement of full enzyme activity (9). The PKC activator $P(BtO)_2$ or the inactive analogue $P(AcO)_2$ was added to intact cells for 2.5 hr before harvesting in some experiments at a concentration of 200 nM. This concentration of $P(BtO)_2$ produced maximal activation of PKC in preliminary dose–response experiments. The reaction was initiated by the addition of 50 μM [^{32}P]ATP, and the reaction was run at 30°C for 10 min. A volume (25 μl) of the phosphorylated protein samples was transferred to Whatman P-81 paper and immersed in 10% trichloroacetic acid. The filters were washed in cold tap water and then in acetone for 3 min, air dried, and counted.

Intact-Cell Phosphorylation Assay. The phosphorylation assay was done with minor modifications of a described method (10). Cells in exponential growth phase at ≈85% confluency were incubated for 60 min at 37°C in phosphate-free IMEM, buffered to pH 7.35 with 20 mM Hepes. Cells were then prelabeled with [^{32}P]orthophosphate at 0.2 mCi/ml (carrier free) for 60 min. Then 10 μM verapamil or 10 μM F_3Pz was added. $P(BtO)_2$ (200 nM) or $P(AcO)_2$ (200 nM) was added 5 min after verapamil or F_3Pz. After 2.5 hr of further incubation, cells were washed in cold phosphate-buffered saline (PBS) three times and solubilized in buffer A with 1% Triton X-100. Protein concentration was determined by the Bradford method (11).

Gel Electrophoresis. Protein samples (16 μg) were separated on 6% or 10% $NaDodSO_4$/polyacrylamide gel electrophoresis based on the method of Laemmli (12). The gels were dried and exposed to film for 18 hr, and densitometry was used for quantitation of phosphoprotein changes; this method of exposure did not exceed the linear range of the film. Phosphorylation was assessed by comparing the intensity of phosphorylation in the 20-kDa region to the intensity of phosphorylation of the whole lane. This value was normalized by comparison of the relative phosphorylation of the 20-kDa region of resistant cells to that of the 20-kDa region in the control line MCF7 WT. Thus a density of 3 units signifies a 3-fold greater intensity of relative 20-kDa phosphorylation in the subject cell line as compared with the MCF7 WT.

Clonogenic Assay. Cells (1000) were plated in IMEM with 10% heat-inactivated fetal calf serum in 30-cm^2 plastic dishes. Twenty-four hr later 10 μM verapamil or 10 μM F_3Pz was added to selected dishes. Five min later $P(AcO)_2$ or $P(BtO)_2$ (both at 200 nM) was added to selected dishes. Then various concentrations of vincristine, doxorubicin, or melphalan were added 5 min thereafter to the above experiments for an additional 2.5 hr. The cells were then washed three times in cold PBS. Cells were refed with fresh IMEM media, and colonies were scored positive 10 days later if greater than 2 × 2 mm. The tumor colony plating efficiency for both lines was ≈10%, and control wells usually had 100 colonies.

Drug Accumulation Assay. Studies were done with modifications of a published method (13). Briefly, MCF7 WT and MCF Dox^R cells were grown in 30-cm^2 plastic wells and were tested in exponential phase growth when they were ≈85% confluent. Cells were washed three times in cold PBS and were then incubated in IMEM with 20 mM Hepes (pH 7.35) but without fetal calf serum. Verapamil or F_3Pz (10 μM) was added to some of the wells. Five min later $P(AcO)_2$ or $P(BtO)_2$ (200 nM) was added to some experimental groups. Five min later [^{3}H]vincristine (final specific activity, 0.117 Ci/mM), [^{14}C]doxorubicin (final specific activity, 1.82 mCi/mM), or [^{14}C]melphalan (final specific activity, 1.39 mCi/mM), diluted in the corresponding unlabeled drug to 10 μM, was added to the above wells for an additional 2.5-hr exposure. The plates were then washed three times in PBS at 4°C, and cells were detached with trypsin and counted in 15 ml of scintillant.

Statistical Analysis. Statistical significance of the experimental results was obtained by the two-sample *t* test.

RESULTS

Differences in Basal and Activated PKC Activity. Basal, unstimulated PKC activity in cell homogenate was 7-fold higher in the MDF Dox^R cells compared with the sensitive WT cells using histone III for substrate (Table 1). Exposure of sensitive or drug-resistant cells to $P(BtO)_2$ led to a 2-fold and 2.5-fold increase in PKC activity, respectively. $P(BtO)_2$ also caused translocation of PKC from soluble-to-particulate fractions. $P(AcO)_2$, an inactive phorbol ester, did not activate PKC or cause translocation (Table 1).

Protein Phosphorylation Changes Induced by Phorbol Ester. Exposure of intact MCF7 WT and Dox^R cells to $P(BtO)_2$ for 2.5 hr led to a general increase in protein phosphorylation in the resistant cell line, as well as a markedly increased labeling of the 20-kDa region in the Dox^R line (Fig. 1). Mean densitometry readings for the 20-kDa region increased from 1.00 in control WT cells to 3.48 units after exposure to $P(BtO)_2$ ($P \leq 0.001$). The baseline 20-kDa phosphoprotein value for the Dox^R line was 3.6 units and increased to 5.02 units (1.4-fold increase) after $P(BtO)_2$ exposure ($P \leq 0.001$). When the cell lines were incubated with the inactive phorbol ester, $P(AcO)_2$, there was no change in the overall phosphoprotein intensity, or in the 20-kDa phosphorylation in either cell line.

We next examined the effects of verapamil and F_3Pz—agents that reverse multidrug resistance—on PKC activity and protein phosphorylation patterns in MCF7 cells. When 10 μM verapamil was added during the 2.5-hr incubation of cells to $P(BtO)_2$, phosphorylation was inhibited. The 20-kDa phosphoprotein value decreased from 3.48 to 1.62 in the WT line, and in the Dox^R line it decreased from 5.02 to 2.02 ($P \leq 0.001$ for each) (Fig. 1). Verapamil alone at 10 μM had no effect on the 20-kDa phosphoprotein in WT cells, but in the Dox^R line it decreased phosphorylation to 1.21 units, or 34%

Table 1. Activation of PKC in MCF7 lines WT and Dox^R

	Total basal PKC activity*	% soluble/particulate PKC activity	Total stimulated PKC activity		% soluble/particulate PKC activity + $P(BtO)_2$
			+ $P(AcO)_2$	+ $P(BtO)_2$	
MCF7 WT	62 ± 11	40/60	54 ± 9	118 ± 8	25/75
MCF7 Dox^R	435 ± 30	40/60	403 ± 18	1090 ± 30	10/90

*Expressed as mean ± SEM pmol/mg of protein per min. Cells were exposed to 200 nM concentrations of $P(BtO)_2$ or $P(AcO)_2$ for 2.5 hr, and PKC activity was measured in cell homogenate and subcellular fractions as described in *Materials and Methods*.

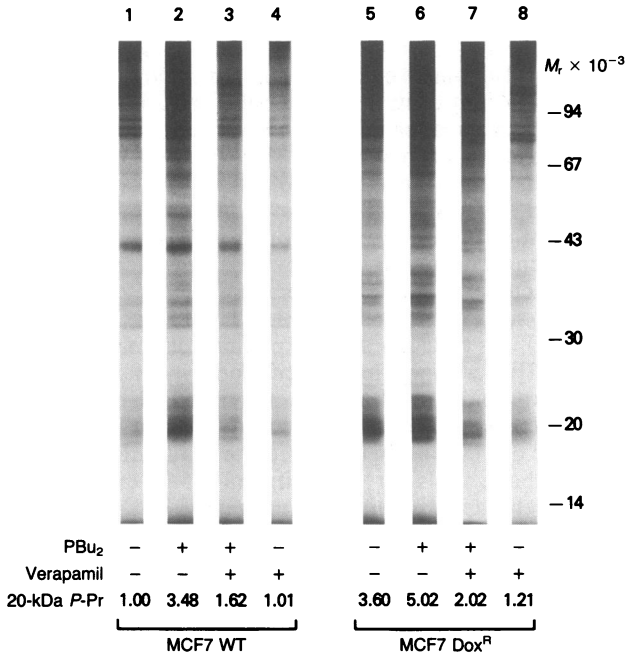

FIG. 1. Effects of phorbol ester and verapamil on protein phosphorylation in MCF7 drug-sensitive (WT) and drug-resistant (Dox^R) cells. Autoradiograms of intact cell phosphorylation experiments from WT (left) and Dox^R (right) are shown. Under each lane is the mean relative phosphorylation (*P*-Pr) of the 20-kDa region in each cell line calculated in units as described. Cells were prelabeled with [^{32}P]orthophosphate (0.2 mCi/ml, carrier free) for 60 min. Then 10 μM verapamil was added, followed in 5 min by 200 nM P(BtO)$_2$ for an additional 2.5 hr. Cells exposed to 200 nM P(AcO)$_2$ did not exhibit changes in phosphorylation and therefore are not shown. Lanes 1 and 5 are baseline WT and Dox^R, respectively; lanes 2 and 6 had 200 nM P(BtO)$_2$ added; lanes 3 and 7 had 200 nM P(BtO)$_2$ and 10 μM verapamil added; and lanes 4 and 8 had only 10 μM verapamil added.

of baseline ($P \leq 0.001$) (Fig. 1). Five μM verapamil had lesser effects in inhibiting 20-kDa phosphorylation. These results indicate that verapamil (which can reverse the MDR phenotype) inhibits baseline phosphorylation of the 20-kDa region in the resistant cells and blocks the increase in phosphorylation induced by P(BtO)$_2$ in both sensitive and resistant cell lines. We found similar results with F_3Pz, which inhibited the increased protein phosphorylation induced by P(BtO)$_2$ in both cell lines (data not shown).

Effects of Phorbol Esters on Drug Sensitivity in Clonogenic Assays. Because the degree of 20-kDa phosphorylation correlates with the level of drug resistance in the MCF7 human breast cancer cell lines (6) and in small-cell lung cancer lines (7), we investigated the possibility that drug sensitivity and drug accumulation could be modulated by phorbol ester-induced PKC activation in intact cells. Table 2 shows the results of clonogenic assays in which the sensitive and resistant cells were preincubated with P(BtO)$_2$ or P(AcO)$_2$ (200 nM) for 2.5 hr and then exposed continuously to vincristine or doxorubicin or melphalan. For the drug-resistant Dox^R line, the baseline IC$_{50}$ of vincristine and doxorubicin were 100-fold higher (20 μM) than the corresponding IC$_{50}$ values of the sensitive WT parent line (0.2 μM). The IC$_{50}$ of melphalan for both cell lines was the same (0.3 μM). However, 200 nM P(BtO)$_2$ increased the IC$_{50}$ to vincristine and to doxorubicin for the WT line from 0.2 μM to 0.8 μM, a 4-fold increase. The mean IC$_{50}$ of the Dox^R line changed from 20 μM to 30 μM in the presence of P(BtO)$_2$. The 4-fold increase in MDR induced by P(BtO)$_2$ in WT cells parallels the 3.48-fold increase in 20-kDa phosphorylation induced by P(BtO)$_2$. Similarly, the 1.5-fold increase in MDR by P(BtO)$_2$ in Dox^R cells approximates the 1.4-fold increase in 20-kDa phosphorylation induced by P(BtO)$_2$. The addition of 10 μM verapamil 5 min before P(BtO)$_2$ blunted the increase in IC$_{50}$ for the WT control cells to 0.4 μM (a 2-fold increase) and completely abrogated the effect of P(BtO)$_2$ in the Dox^R cells, reducing the IC$_{50}$ below baseline to 5 μM. When verapamil was added in the absence of P(BtO)$_2$ for 2.5 hr, the mean IC$_{50}$ values of doxorubicin and vinblastine did not change in the WT cells but decreased in the Dox^R cells from the baseline 20 μM to 2 μM. F_3Pz similarly reversed the effects of P(BtO)$_2$ (data not shown). P(BtO)$_2$ with or without verapamil did not alter the IC$_{50}$ values when the cells were exposed to melphalan, an alkylating agent not affected by MDR, suggesting that P(BtO)$_2$ and verapamil affected a process specific for natural-product drugs. The addition of the inactive ester, 200 nM P(AcO)$_2$, did not change the mean IC$_{50}$ values of vincristine, doxorubicin, or melphalan for either line. These experiments show that P(BtO)$_2$ increases (*i*) PKC activity, (*ii*) the level of MDR, and (*iii*) the phosphorylation of the 20-kDa protein. The latter two effects of P(BtO)$_2$ are inhibited by verapamil and F_3Pz.

Effects of Phorbol Esters on Growth Rates. Phorbol esters have been reported to inhibit the proliferation of MCF7 cells during a 4-day coincubation (14). This decrease in cell growth rate might have an effect on drug sensitivity. P(BtO)$_2$ and P(AcO)$_2$ were added to exponentially growing WT and Dox^R cells for 2.5 hr, and cells were then washed three times in cold PBS and refed with drug-free media. Cell counts of flasks with <85% confluency were done on days 1 through 7 and

Table 2. Effects of P(AcO)$_2$ or P(BtO)$_2$ with or without verapamil on drug-sensitivity clonogenic assay

	IC$_{50}$ for MCF7 WT, μM		IC$_{50}$ for MCF7 Dox^R, μM	
	Vincristine	Doxorubicin	Vincristine	Doxorubicin
Control	0.2 ± 0.04	0.2 ± 0.03	20 ± 0.9	20 ± 1.0
P(AcO)$_2$ (200 nM)	0.2 ± 0.06*	0.2 ± 0.08*	20 ± 1.4*	20 ± 1.8*
P(BtO)$_2$ (200 nM)	0.8 ± 0.04†	0.8 ± 0.06†	30 ± 1.8†	30 ± 1.9†
P(BtO)$_2$/verapamil (10 μM)	0.4 ± 0.06‡	0.4 ± 0.05‡	5 ± 0.8†	5 ± 0.9†
Verapamil (10 μM)	0.2 ± 0.05*	0.2 ± 0.07*	2 ± 0.6†	2 ± 0.8†

Values represent mean ± SEM of three experiments done in triplicate. Briefly, 24 hr after cell plating, verapamil was added, followed in 5 min by P(AcO)$_2$ or P(BtO)$_2$; then 5 min later a range of concentrations of vincristine, doxorubicin, or melphalan were added for a 2.5-hr incubation. Cells were subsequently washed in cold PBS three times and were refed with drug-free media. Colonies were stained and scored positive 10 days later if larger than 2 × 2 mm. Experiments with F_3Pz had results similar to those with verapamil. Studies with melphalan showed no difference between control and groups exposed to P(BtO)$_2$, P(AcO)$_2$, verapamil, and F_3Pz (all had mean IC$_{50}$ values of 0.3 μM).
*$P > 0.05$.
†$P < 0.001$.
‡$P \leq 0.05$.

compared to counts of control cells not exposed to phorbol ester. We found no evidence that $P(BtO)_2$ or $P(AcO)_2$ inhibited the growth rates of these cells and conclude that short-term phorbol ester exposure did not increase MDR through cell kinetic effects.

Phorbol Ester-Induced Changes in Drug Accumulation. A common characteristic of MDR cells is their net decreased intracellular accumulation of natural-product antineoplastic drug due to increased drug efflux. Because PKC activation is known to increase exocytosis of many cellular products (15), we examined the effects of phorbol esters on accumulation of doxorubicin and vincristine. Table 3 shows the results of drug accumulation studies with [^{3}H]vincristine and [^{14}C]doxorubicin in sensitive and resistant cell lines during a 2.5-hr exposure to these agents in the absence or presence of $P(BtO)_2$ or $P(AcO)_2$. Mean baseline accumulation of vincristine in the MCF7 WT cells was 357 pmol per 10^6 cells, whereas the MCF7 Dox^R cells accumulated 52 pmol per 10^6 cells. Addition of the inactive ester $P(AcO)_2$ did not significantly change baseline accumulation of any of the three drugs in either line. However, $P(BtO)_2$ at 200 nM decreased accumulation of vincristine to 60% and 88% of control in the WT and Dox^R cells, respectively. Verapamil (10 μM) added 5 min before $P(BtO)_2$ restored [^{3}H]vincristine accumulation to 89% and 165% of baseline drug levels in WT and Dox^R, respectively. Verapamil alone had no effect on the [^{3}H]vincristine accumulation in the WT cells, but further increased accumulation in the Dox^R cells to 227% above the baseline drug accumulation. F_3Pz (10 μM) had similar effects to verapamil in the above studies (data not shown).

$P(BtO)_2$ similarly decreased accumulation of [^{14}C]doxorubicin more in MCF7 WT than in Dox^R cells, and its effects were reversed by verapamil (Table 3) or F_3Pz. The $P(BtO)_2$ effect seems to be specific for agents involved in MDR because [^{14}C]melphalan accumulation was not affected by $P(BtO)_2$. These effects on drug accumulation parallel the effects of $P(BtO)_2$ with or without verapamil or F_3Pz on drug sensitivity and 20-kDa phosphorylation.

DISCUSSION

These studies demonstrate that PKC activation by $P(BtO)_2$ can induce MDR in sensitive control MCF7 cells and can further increase resistance in the drug-resistant mutant MCF7 cells. $P(BtO)_2$ exposure was associated with decreased intracellular accumulation of drug and with increased phosphorylation of the 20-kDa protein(s) that we have found in MDR human breast and small-cell lung cancer lines. These findings suggest that phorbol ester may cause decreased accumulation of drug by inducing phosphorylation of a drug efflux pump or carrier protein. There is considerable precedent for a regulatory role of PKC in transport processes. PKC activation is associated with increased exocytosis of histamine and arachidonate in platelets, amylase and insulin secretion in pancreatic cells, lysosomal enzyme release in neutrophils, catecholamine release from adrenal medullary cells, aldosterone secretion from adrenal cortex, and dopamine release from neurons (15). Membrane proteins that transport Ca^{2+}, Na^+, K^+, H^+, and glucose are proposed substrates for PKC and may be regulated by phosphorylation (15). Thus, it is possible that decreased anticancer drug accumulation in resistant cells may be related to a PKC-regulated mechanism.

Previous experiments with human tumor cells have indicated that phorbol esters influence both sensitivity to natural-product-type drugs and drug accumulation. Phorbol esters induced resistance to colchicine in lymphocytes from patients with chronic lymphocytic leukemia (16).

Phorbol esters also transiently increased resistance (2-fold) of drug-sensitive human KB (epidermoid) carcinoma cells to vincristine and the epipodophyllotoxin VP-16 (17), an effect associated with a 30% decrease of intracellular drug accumulation, and this decrease was antagonized by verapamil. Phorbol esters have also been shown to decrease vincristine accumulation by 40% in drug-sensitive P388 murine leukemia cells (18). The isoquinoline sulfonamide H-7, which inhibits PKC activity, reversed the decreased intracellular accumulation of vincristine in these P388 cells induced by phorbol esters. However, in none of these studies were changes in protein phosphorylation examined.

In the present studies, phorbol ester produced an increase in PKC activity in both drug-sensitive and drug-resistant cells and induced the phosphorylation of a 20-kDa protein in drug-sensitive cells; the changes in 20-kDa protein phosphorylation paralleled levels of drug sensitivity as modulated by phorbol esters, verapamil, and F_3Pz. The intensity of the phosphorylation of the 20-kDa protein was the only consistent finding that correlated with multidrug resistance in all of human breast and small-cell lung cancer lines that we studied (6, 7). The 20-kDa protein was detected by ^{32}P labeling but not detected by silver staining or [^{35}S]methionine labeling of cells in 2-dimensional gel electrophoresis (unpublished work). Thus, the phosphorylation of the protein was increased,

Table 3. [^{3}H]Vincristine and [^{14}C]doxorubicin accumulation in MCF7 WT and Dox^R cells: Effect of $P(BtO)_2$, $P(AcO)_2$, and verapamil

	MCF7 WT		MCF7 Dox^R	
	pmol per 10^6 cells	% control drug	pmol per 10^6 cells	% control drug
Vincristine	357 ± 12	—	52 ± 2	—
+ $P(AcO)_2$	349 ± 17*	98	53 ± 2*	100
+ $P(BtO)_2$	217 ± 9†	60	46 ± 2‡	88
+ $P(BtO)_2$ + verapamil	316 ± 11‡	89	86 ± 4†	165
+ Verapamil	359 ± 8*	100	118 ± 9†	227
Doxorubicin	210 ± 18	—	73 ± 11	—
+ $P(AcO)_2$	206 ± 20*	98	72 ± 10*	99
+ $P(BtO)_2$	138 ± 12†	65	65 ± 4*	89
+ $P(BtO)_2$ + verapamil	201 ± 14*	96	83 ± 3*	114
+ Verapamil	214 ± 12*	102	110 ± 10†	150

Ten micromolar verapamil was added, and then 5 min later 200 nM $P(BtO)_2$ or $P(AcO)_2$ was added. Five minutes later radioactive vincristine, doxorubicin, and melphalan diluted to 10 μM in nonradioactive drug were added for an additional 2.5 hr. Each value is a mean ± SEM of three experiments in triplicate. Experimental protocol followed a published method (13). The [^{14}C]melphalan accumulation studies showed no difference between control and the group exposed to $P(BtO)_2$, $P(AcO)_2$, verapamil, and F_3Pz (191 ± 10 pmol per 10^6 cells). F_3Pz produced results similar to verapamil.

*$P > 0.05$.

†$P < 0.001$.

‡$P \leq 0.05$.

whereas the content of the phosphorylated protein may not be increased. The protein was found solely in the particulate fraction. The pI of the 20-kDa phosphoprotein was between 7.5 and 7.8, and serine was the only residue phosphorylated in 2-dimensional phosphoamino acid analysis (unpublished work). Meyers and Biedler have described a 19-kDa protein that is overexpressed in MDR Chinese hamster cells (19). It is unlikely to be homologous to the 20-kDa phosphoprotein because its pI is 5.7 and it is cytoplasmic. Batist *et al.* (20) have found increased amounts of a 22-kDa anionic glutathione-*S*-transferase in the MCF7 Dox^R cells described in this paper. This enzyme is cytoplasmic and has a pI of 4.7, and thus it is unlikely to be the 20-kDa phosphoprotein. Myosin light chain has a M_r of 20 kDa, but it has a pI of 5.7 and is found mainly in the cytoplasm (21). Thus, the identity and function of this protein is unknown.

The relatively greater effect of $P(BtO)_2$ on the WT control cells insofar as increased 20-kDa phosphorylation, decreased drug accumulation, and induction of MDR as compared to the Dox^R cells suggests that the mechanism(s) producing MDR was inducible in the sensitive cells but may be near maximal activity in the resistant cells. The mechanism(s) by which verapamil and F_3Pz inhibited increased protein phosphorylation, decreased intracellular drug accumulation, and increased drug resistance associated with phorbol activation of PKC is not clear. It has been shown that purified PKC can be inhibited *in vitro* by 50% (IC_{50}) with 500 μM verapamil (5) and 60 μM F_3Pz (22). Experiments in our lab with purified rat brain PKC have demonstrated that verapamil and F_3Pz at 10 μM do not directly inhibit phosphorylation of histone-IIIs by PKC in cell-free systems when the reaction is stimulated by phosphatidylserine, diolene, and $CaCl_2$ (data not shown).

The combination of PKC inhibitors with anticancer drugs derived from natural products may prove useful in treating neoplasms with the multidrug-resistant phenotype. It has been shown that agents such as doxorubicin (22) and tamoxifen (23) can inhibit PKC at micromolar concentrations. Perhaps the combination of these agents with classic or new inhibitors of PKC such as the isoquinoline sulfonamides or staurosporine (24) may circumvent MDR and open new approaches to anticancer therapy.

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