0026-895X/92/061008-08$03.00/0

MOLECULAR PHARMACOLOGY, 41:1008–1015

Regulation by Phorbol Ester and Protein Kinase C Inhibitors, and by a Protein Phosphatase Inhibitor (Okadaic Acid), of P-Glycoprotein Phosphorylation and Relationship to Drug Accumulation in Multidrug-Resistant Human KB Cells

TIMOTHY C. CHAMBERS, BIN ZHENG, and J. F. KUO

Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Received November 26, 1991; Accepted March 11, 1992

SUMMARY

Covalent modification by phosphorylation is a characteristic of the P-glycoproteins expressed in multidrug-resistant cells. This report describes analysis of P-glycoprotein phosphorylation in multidrug-resistant human KB-V1 cells and a study of the relationship of phosphorylation and drug accumulation. In isolated membranes, phosphorylation of P-glycoprotein by purified protein kinase C (PKC) was rapid, and time-dependent dephosphorylation was inhibited by okadaic acid, an inhibitor of type 1 and type 2A protein phosphatases. In ^{32}P-labeled intact KB-V1 cells, P-glycoprotein phosphorylation was stimulated by both 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of PKC, and okadaic acid. Two-dimensional thin layer tryptic phosphopeptide maps indicated that the sites of phosphorylation were similar in control, TPA-treated, and okadaic acid-treated cells and that they corresponded to those phosphorylated by PKC *in vitro*. The protein kinase inhibitor staurosporine, and the PKC-selective inhibitors calphostin C and the alkyl-lysophospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, inhibited P-glycoprotein phosphorylation *in vitro* and in intact cells. Drug accumulation assays demonstrated that in KB-V1 cells TPA caused a decrease, whereas staurosporine and calphostin C caused an increase, in accumulation of [^{3}H]vinblastine. These compounds did not significantly alter [^{3}H]vinblastine levels in drug-sensitive KB-3 cells. These results suggest that PKC is chiefly responsible for P-glycoprotein phosphorylation in KB-V1 cells, that membrane-associated protein phosphatases 1 and 2A are active in dephosphorylation of P-glycoprotein, and that phosphorylation of P-glycoprotein may be an important mechanism for modulation of drug-pumping activity.

The MDR phenotype is closely associated with amplification of the *mdr* gene and overexpression of its product, P-glycoprotein, a plasma membrane glycoprotein of M_r 150,000–180,000 (see Ref. 1 for recent review). Compelling evidence for a key role of P-glycoprotein in MDR has come from many sources, most notably transfection studies, where expression of the *mdr1* gene or cDNA confers MDR on the recipient cells (2–6). P-glycoprotein transports structurally diverse drugs out of resistant cells, through its ability to act as an energy-dependent drug efflux pump of broad specificity. This conclusion is based on studies showing that P-glycoprotein possesses sequence homology with bacterial transport proteins (7, 8), ATP binding (9) and hydrolysis (10) activities, drug binding (11) and efflux (12) properties, and the ability to bind certain agents that reverse resistance, such as verapamil (13, 14) and cyclosporin (15).

Covalent modification by phosphorylation is an established characteristic of P-glycoproteins, occurring in cells selected for resistance (16–23) and in cells transfected with *mdr1* cDNA (24) or the *mdr1* gene (25). The apparent universal occurrence of this covalent modification raises the possibility that phosphorylation may be a major regulatory mechanism for P-glycoprotein function. Thus, new approaches for circumvention of resistance may be based on pharmacological manipulation of phosphorylation levels. Information concerning the identity of the protein kinases and phosphatases acting on P-glycoprotein is, therefore, of considerable significance. The calcium-activated, phospholipid-dependent protein kinase, PKC, has been implicated, because the PKC activator TPA stimulates P-glycoprotein phosphorylation in several MDR cell lines (19, 21,

This work was supported by United States Public Health Service Research Grants CA-36777 and HL-15696 and by American Cancer Society Grant CH-513.

ABBREVIATIONS: MDR, multidrug resistance or multidrug resistant; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-*O*-acetate; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PP1 and PP2A, protein phosphatases 1 and 2A, respectively; IC_{50}, concentration causing 50% inhibition; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

25) and PKC phosphorylates P-glycoprotein *in vitro* (21, 26). In addition, increased PKC activity is generally associated with the development of MDR (21, 26–30). P-glycoprotein is also phosphorylated *in vitro* by cAMP-dependent protein kinase (31) and by a calcium-independent, phospholipid-dependent protein kinase (32). However, detailed comparisons, by, for example, two-dimensional thin layer techniques, of *in vitro* and *in vivo* phosphopeptides derived from P-glycoprotein have not been reported. Thus, the contribution of different kinases to the overall level of phosphorylation of P-glycoprotein in a given MDR cell line is not presently clear. Further, the types of phosphatases that dephosphorylate P-glycoprotein have not been characterized.

Studies by Fine *et al.* (28) indicated that exposure to phorbol esters increased the drug resistance of MDR human breast cancer cells. In preliminary studies using MDR human KB cells treated with TPA, we observed a correlation between P-glycoprotein phosphorylation and reduced drug accumulation (33). Recently, inhibition of P-glycoprotein phosphorylation by staurosporine in vincristine-resistant HL60 cells has been shown to be associated with increased drug accumulation (23). Thus, evidence from several different MDR cell lines based on the use of a single agent (i.e., TPA or staurosporine) appear to indicate that phosphorylation stimulates the drug-pumping activity of P-glycoprotein. A more complete study, in which the relationship between phosphorylation and drug accumulation is examined in response to both activators and inhibitors of phosphorylation, seemed warranted. In the present work, we utilized the PKC activator TPA, the protein phosphatase inhibitor okadaic acid, the PKC-selective inhibitors calphostin C and ET-18-OCH_3, and the nonselective protein kinase inhibitor staurosporine, to examine P-glycoprotein phosphorylation *in vitro* and to manipulate pharmacologically levels of P-glycoprotein phosphorylation in intact MDR cells. The results suggest that PKC is chiefly responsible for phosphorylation and that PP1 and PP2A have roles in dephosphorylation of P-glycoprotein. In order to clarify the relationship between P-glycoprotein phosphorylation and function, we studied drug accumulation in response to these agents, in both drug-resistant and drug-sensitive cells.

Experimental Procedures

Materials. [^{3}H]Vinblastine (12 Ci/mmol) was obtained from Amersham Corp.; [^{32}P]orthophosphate (10 mCi/ml) was from ICN Radiochemicals; okadaic acid was from Moana BioProducts; ET-18-OCH_3 was from Calbiochem; calphostin C and staurosporine were from Kamiya Biomedical Co.; monoclonal antibody C219 for P-glycoprotein was from Centocor Diagnostics; and Protein A-Sepharose, PS (bovine brain), and TPA were obtained from Sigma. PKC from pig brain was purified to about 50% homogeneity and devoid of other protein kinases through a phenyl-Sepharose step (34). [γ-^{32}P]ATP was prepared as described (35).

Cell culture. Human KB carcinoma cell lines were used in this study (36). The drug-sensitive parental cell line KB-3 was maintained in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose, 10% fetal bovine serum, and 10 mM HEPES, pH 7.3, and the drug-resistant cell line KB-V1 in the same medium containing 1 μg/ml vinblastine. For metabolic labeling with [^{32}P]phosphate, cell cultures in 75-cm^2 flasks were incubated in 6 ml of phosphate-free medium containing [^{32}P]orthophosphate (0.1–0.2 mCi/ml), for periods of 2–4 hr.

Preparation and phosphorylation of membranes. Membrane vesicles were prepared as described (11). Standard phosphorylation reaction mixtures (0.1–0.5 ml) contained membranes (1 mg/ml) in 20 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 0.25 M sucrose, 10 μM [γ-^{32}P]ATP (2–3 × 10^3 cpm/pmol), with or without 0.2 mM $CaCl_2$, 50 μg/ml PS, 0.2 μM okadaic acid, and 0.1 unit of PKC (where 1 unit = 1 μmol of phosphate transferred to H1 histone at 30° in 1 min). Reactions were performed at 30° for periods of 2–120 min and were terminated by the addition of SDS sample buffer (37).

Cell extraction, immunoprecipitation, and SDS-PAGE. After ^{32}P-labeling and treatment, cells were rinsed and gently scraped into 5 ml of ice-cold PBS. This and all subsequent solutions contained the following: 0.2 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 0.2 mM sodium vanadate, and 20 nM okadaic acid. After centrifugation (500 × *g*, 5 min), individual cell pellets were suspended in 1 ml of radioimmune precipitation buffer (10 mM Tris·HCl, pH 7.2, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) and, after 10 min on ice, the suspension was centrifuged (100,000 × *g*, 30 min). Trichloroacetic acid-precipitable radioactivity was determined in 10-μl aliquots, and equivalent amounts of radioactivity from each sample (4–5 × 10^6 cpm, 300–500 μg of protein) were mixed for 2 hr with P-glycoprotein monoclonal antibody C219 (1 μg/100 μg of sample protein). Protein A-Sepharose (0.2 ml, 25%, w/v, in PBS) was then added and mixed for an additional 30 min; precipitates were collected by centrifugation and washed once with 1 ml of 1 M NaCl, 1% Nonidet P-40, and three times with 1 ml of radioimmune precipitation buffer containing 1 M urea. The precipitate was finally incubated at 30° for 10 min in 0.1 ml of SDS sample buffer (37), and Protein A-Sepharose was removed by centrifugation. Eluted material was analyzed by SDS-PAGE (37) using 6% acrylamide separation gels. Gels were stained with Coomassie Blue, destained, dried, and exposed to Kodak XAR film at −70°.

Two-dimensional phosphopeptide mapping. Gel slices containing phosphorylated P-glycoprotein were soaked in 5 ml of 0.2 M NH_4HCO_3, pH 8.0, for 15 min and then incubated for 16 hr at 37° in 1 ml of 0.2 M NH_4HCO_3, pH 8.0, containing 50 μg of L-1-tosylamido-phenylethyl chloromethyl ketone-treated trypsin. Lyophilized preparations of the eluted peptides were subjected to two-dimensional thin layer separation, as described (38). Briefly, peptides were dissolved in 10 μl of electrophoresis buffer (pyridine/glacial acetic acid/water, 1:10:89, pH 3.7), applied to thin layer cellulose plates, and electrophoresed toward the cathode at 450 V for 4 hr. Second-dimension ascending chromatography was performed with a solvent system of *n*-butanol/pyridine/glacial acetic acid/water (50:33:1:40), and autoradiographs were taken with Kodak XAR film at −70°. Quantitation was performed by densitometric scanning of the autoradiographic film using an E-C Apparatus Corp. densitometer.

Drug accumulation studies. Cellular accumulation of [^{3}H]vinblastine was performed essentially as described previously (39), with modifications as follows. KB-3 or KB-V1 cells (in vinblastine-free medium) were plated in 24-well plates (3 × 10^5 cells/well). In some experiments, the cells were preincubated with the compound of interest dissolved in dimethylsulfoxide (at a maximum final concentration of 0.05%), with controls receiving solvent alone. The assay was initiated by replacing the medium with 0.5 ml of serum-free growth medium, adjusted to pH 7.35 with 50 mM HEPES, containing the compound of interest, [^{3}H]vinblastine (26–36 nM, 50,000–70,000 cpm), in the absence or presence of 20 μM verapamil. After 1 hr, the medium was removed and individual wells were washed rapidly three times with 1 ml of ice-cold PBS. Up to six wells were used for each experimental condition. The cells in three or four of the wells were detached with trypsin, and radioactivity was determined by scintillation counting. The cells in the other wells were dissolved in 0.5 ml of 0.5 N NaOH, and the protein concentration was determined in 10-μl aliquots, by the method of Bradford (40), using bovine serum albumin as a standard.

Results

Increased P-glycoprotein phosphorylation by PKC, TPA, and okadaic acid. In the first set of experiments, we used an *in vitro* membrane phosphorylation system to investi-

gate P-glycoprotein phosphorylation/dephosphorylation. Fig. 1A (*lanes 1–4*) shows calcium/phospholipid-dependent phosphorylation of membrane-bound P-glycoprotein by PKC during a 2-min incubation. P-glycoprotein (M_r 150,000) is the major PKC substrate present, its identity having been confirmed previously by immunoprecipitation with the P-glycoprotein-specific monoclonal antibody C219 (21). Okadaic acid, an inhibitor of PP1 and PP2A (41), appeared to increase slightly the basal (Fig. 1A, *lane 5*) and PKC-catalyzed phosphorylation (Fig. 1A, *lane 6*) of P-glycoprotein after a 2-min incubation. To investigate this in more detail, a time course of phosphorylation was conducted. As shown in Fig. 1B (*lanes 1–5*), PKC-catalyzed phosphorylation of P-glycoprotein was maximal at ≤5 min and progressively decreased from 5 to 120 min. The Coomassie Blue staining pattern of proteins, including the intensity of the P-glycoprotein band, was invariant in each lane (data not shown). This excluded the possibility of loss of radiolabeling due to protein degradation and suggested the presence of membrane-associated protein phosphatase activity acting on P-glycoprotein. This was confirmed by conducting the same time course in the presence of 200 nM okadaic acid (Fig. 1B, *lanes 6–10*). In this case, dephosphorylation of P-glycoprotein was nearly completely prevented, thus implicating PP1 and/or PP2A in the dephosphorylation reaction.

P-Glycoprotein phosphorylation was analyzed in intact cells by immunoprecipitation of extracts prepared from ^{32}P-labeled KB-V1 cells after treatment with okadaic acid (100 nM, 1 hr), TPA (100 nM, 1 hr), or both (Fig. 2). Okadaic acid caused a modest but reproducible increase in phosphorylation (Fig. 2, *lane 2*), estimated to be 35 ± 5% (four experiments) by excision of the gel band and determination of radioactivity, relative to the control (Fig. 2, *lane 1*). This increase in phosphorylation induced by okadaic acid appeared to be the maximum achievable, because no further increase was observed at higher concentrations (up to 300 nM) or with longer times of treatment (up to 4 hr, the duration of $^{32}P_i$ labeling) (data not shown). Thus, a disparity exists between the effectiveness of okadaic acid *in vitro* (Fig. 1) and in intact cells (Fig. 2). Preliminary evidence suggests that this may be due to a reduced intracellular concentration of the toxin, possibly involving P-glycoprotein-mediated transport.[1] As observed previously (21), TPA induced a marked (2–3-fold) increase in phosphorylation (Fig. 2, *lane 3*). Despite different modes of action, okadaic acid and TPA apparently did not produce additive effects on phosphorylation (Fig. 2, *lane 4*). No protein radiolabeling was observed in immunoprecipitates of extracts prepared from ^{32}P-labeled KB-3 cells treated without or with TPA and okadaic acid (Fig. 2, *lanes 5* and *6*).

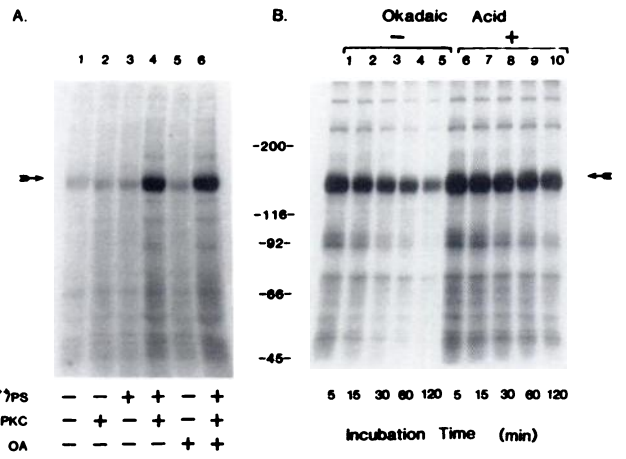

Fig. 1. Phosphorylation of membrane-bound P-glycoprotein by PKC *in vitro* and inhibition of dephosphorylation by okadaic acid. A, Membranes from KB-V1 cells were incubated for 2 min at 30° in standard phosphorylation reaction mixtures, as described in Experimental Procedures. Reactions were performed in the absence or presence of Ca^{2+}/PS, PKC, and okadaic acid (*OA*) (200 nM), as indicated. B, Membranes from KB-V1 cells were incubated, for the times indicated, in complete phosphorylation reaction mixtures containing Ca^{2+}/PS and PKC, in the absence or presence of 200 nM okadaic acid. Autoradiographs of 6% acrylamide-SDS gels are shown. Aliquots containing 50 μg of protein were run in each lane. Molecular masses of standard proteins (in kDa) are indicated in the *center*. *Arrows*, P-glycoprotein (150 kDa). The results shown are representative of at least three separate experiments.

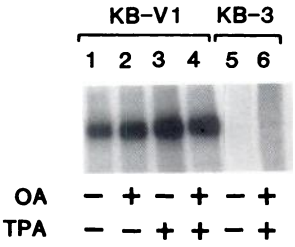

Fig. 2. Effects of okadaic acid and TPA on phosphorylation of P-glycoprotein in intact cells. Cultures of KB-V1 or KB-3 cells were labeled with $^{32}P_i$ (0.15 mCi/ml) for 4 hr at 37°. During the final hour of labeling, okadaic acid (100 nM), TPA (100 nM), or both were added as indicated. Cell extracts were prepared and immunoprecipitated with monoclonal antibody C219, using equivalent amounts of acid-precipitable radioactivity (5×10^6 cpm) from each extract, and samples were subjected to SDS-PAGE and autoradiography. Similar results have been obtained in four separate experiments. *OA*, okadaic acid.

Phosphopeptide mapping. Two-dimensional thin layer chromatographic maps of tryptic phosphopeptides derived from P-glycoprotein indicated that the sites of phosphorylation were similar in control, TPA-treated, and okadaic acid-treated KB-V1 cells (Fig. 3, A–C). Thus, the increased overall level of phosphorylation induced by TPA or okadaic acid (Fig. 2) does not appear to involve phosphorylation of new sites. Furthermore, the same three phosphopeptides were obtained from P-glycoprotein phosphorylated *in vitro* by PKC, using membranes of KB-V1 cells (Fig. 3D). The fact that the three phosphopeptides corresponded in each case was confirmed by mixing digests before analysis (data not shown). Quantitation of the distribution of radioactivity among the three phosphopeptides (see legend to Fig. 3) indicated that *in vitro* PKC appeared to enhance phosphorylation of the lower two phosphopeptides slightly (Fig. 3D), compared with the more even distribution of radioactivity among the three phosphopeptides derived from P-glycoprotein isolated from intact cells (Fig. 3, A–C). Despite this difference in distribution of radioactivity, the similarities of the individual phosphopeptide maps provide strong evidence for the identification of PKC as the major kinase acting on P-glycoprotein in KB-V1 cells. When KB-V1 membranes were incubated under phosphorylation conditions in the absence of added PKC, a low level of endogenous phosphorylation was observed (e.g., Fig. 1, *lane 1*). Although the amount of radioactivity incorporated under these conditions was insufficient to permit phosphopeptide mapping, previous results have indicated that this endogenous phosphorylation is catalyzed by membrane-associated PKC (21).

[1] Dr. Timothy C. Chambers, and J. F. Kuo, unpublished observations.

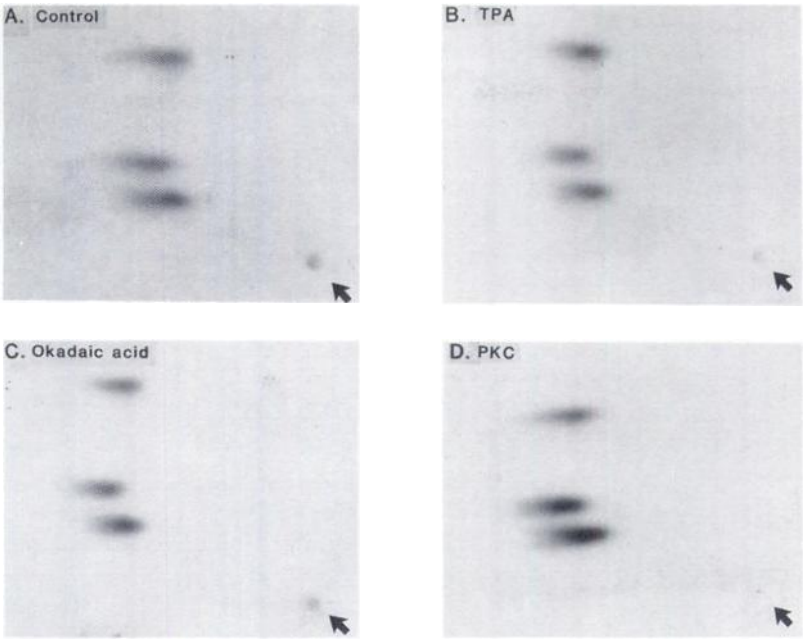

Fig. 3. Autoradiography of two-dimensional thin layer tryptic peptide maps of phosphorylated P-glycoprotein. A-C, P-glycoprotein isolated by immunoprecipitation and SDS-PAGE from extracts of ^{32}P-labeled intact KB-V1 cells (see Fig. 2); A, control; B, 100 nM TPA; C, 100 nM okadaic acid. D, P-glycoprotein isolated by SDS-PAGE after *in vitro* phosphorylation of KB-V1 cell membranes by PKC (see Fig. 1). Amounts of radioactivity subjected to analyses were 350 cpm (A–C) and 500 cpm (D). First-dimension electrophoresis was from *right* to *left*, and second-dimension chromatography was from *bottom* to *top*. *Arrows*, origins. The distribution of radioactivity among the three phosphopeptides (in the order of lower, middle, and upper phosphopeptide), determined as described in Experimental Procedures, was as follows: A, 39%, 29%, and 32%; B, 39%, 29%, and 32%; C, 42%, 31%, and 27%; D, 47%, 36%, and 17%. Essentially identical results have been obtained in two additional experiments.

Effects of TPA and okadaic acid on vinblastine accumulation. The effects of TPA on basal and verapamil-induced vinblastine accumulation in both KB-V1 and KB-3 cells were examined and compared (Fig. 4). We have shown previously that verapamil, which induces higher levels of vinblastine accumulation through competition for P-glycoprotein drug-binding sites (13, 14), does not significantly increase P-glycoprotein phosphorylation, relative to TPA (21). In KB-V1 cells, treatment with TPA caused a marked decrease, up to 75%, in verapamil-induced vinblastine accumulation. Half-maximal effects were observed at ~28 nM TPA and greatest effects at >100 nM, conditions that corresponded to highest levels of TPA-induced phosphorylation of P-glycoprotein (Fig. 2). The low basal level of vinblastine that accumulated in this highly resistant cell line in the absence of verapamil (0.76 ± 0.05 pmol/mg) was also reduced after TPA (300 nM) treatment (to 0.48 ± 0.03 pmol/mg), with half-maximal effects being observed at 100 nM. It is evident from comparison of the two curves that verapamil-induced vinblastine accumulation is quantitatively reversed by TPA under these conditions. In contrast, no clear relationship between vinblastine accumulation and TPA treatment was observed in drug-sensitive KB-3 cells, although verapamil caused a small reduction in the level of vinblastine accumulated, as observed previously (39).

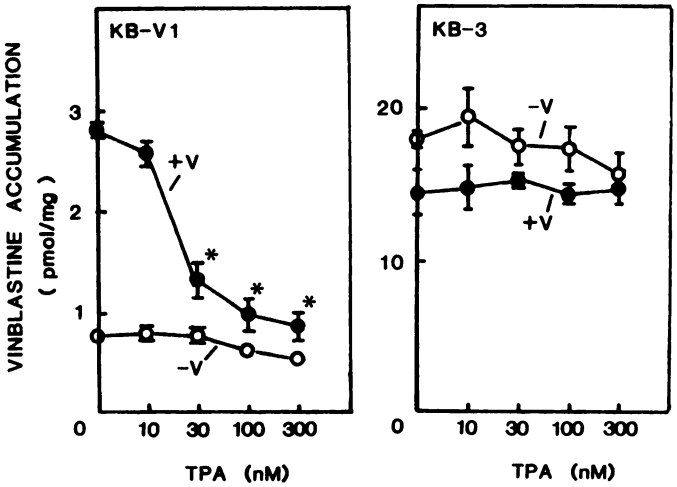

Fig. 4. Effect of TPA on vinblastine accumulation. KB-V1 or KB-3 cells were incubated for 1 hr at 37° with 26 nM [^{3}H]vinblastine, with or without 20 μM verapamil (*V*), at the indicated concentrations of TPA. Data shown are mean ± standard error (three determinations) and are representative of three separate experiments. *, Significantly different from control in the absence of TPA ($p < 0.005$).

The accumulation of vinblastine in both cell lines after exposure to okadaic acid was examined. However, experimental conditions were limited because okadaic acid was found to be cytotoxic, particularly in KB-3 cells, which were much more sensitive than KB-V1 cells to the effects of the toxin.[1] This may be related to a decreased accumulation of toxin in the resistant cells, alluded to above. We did find that treatment of KB-V1 cells for 1 hr with 100 nM okadaic acid, i.e., conditions as in Fig. 2, did not significantly alter basal or verapamil-induced accumulation of vinblastine (data not shown). We conclude that, whereas large increases in phosphorylation, i.e., by TPA, result in a significant decrease in drug accumulation, the small increase in phosphorylation produced by okadaic acid does not result in a measurable change in vinblastine accumulation under these experimental conditions. Demonstration of a physiological role of dephosphorylation of P-glycoprotein may, thus, require the use of alternative phosphatase inhibitors that are more effective in the drug-resistant cells.

Inhibition of P-glycoprotein phosphorylation. To complement the studies described above, we sought to identify inhibitors of phosphorylation. Initial screening was based on inhibition of PKC-catalyzed phosphorylation of P-glycoprotein in our *in vitro* membrane phosphorylation system. As shown in Fig. 5 (*lanes 1–7*), the nonselective protein kinase inhibitor staurosporine (42) and the PKC-selective inhibitors ET-18-OCH_3 (43) and calphostin C (44), with reported IC_{50} values for PKC of 3 nM, 8 μM, and 50 nM, respectively, were all effective, albeit to different degrees, in this assay. As a control, phosphorylation studies were also conducted with membranes from KB-3 cells, where P-glycoprotein is absent (Fig. 5, *lanes 8–11*).

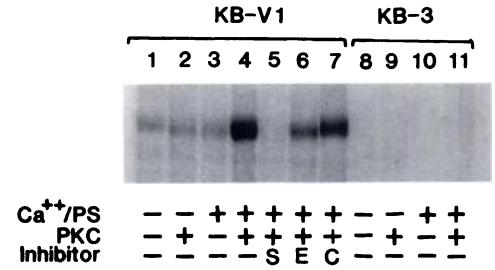

Fig. 5. Effect of PKC inhibitors on PKC-catalyzed phosphorylation of P-glycoprotein *in vitro*. Membranes from KB-V1 cells or KB-3 cells were incubated for 5 min at 30° in standard phosphorylation reaction mixtures containing Ca^{2+}/PS, PKC, and the PKC inhibitors 0.5 μM staurosporine (*S*), 50 μM ET-18-OCH_3 (*E*), or 5 μM calphostin C (*C*), as indicated. Aliquots containing 50 μg of protein were run in each lane. Only the portion of the autoradiograph containing P-glycoprotein is shown. This experiment has been repeated with the same results.

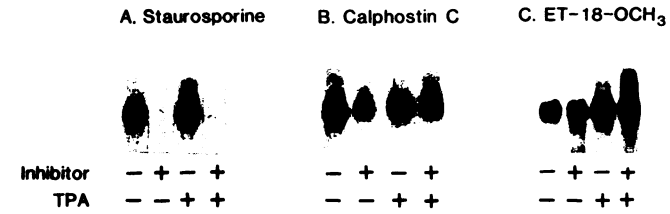

Fig. 6. Effect of PKC inhibitors on P-glycoprotein phosphorylation in intact cells. Cultures of KB-V1 cells were labeled for 2.5 hr with 0.1 mCi/ml $^{32}P_i$. During the final hour of labeling, cells were treated as indicated with either 0.5 μM staurosporine (A), 5 μM calphostin C (B), or 5 μM ET-18-OCH_3 (C). During the final 30 min of labeling, TPA (100 nM) was added where indicated. Cell extracts were prepared, equivalent amounts of acid-precipitable radioactivity (4.5×10^6 cpm) were subjected to immunoprecipitation, and samples were analyzed by SDS-PAGE and autoradiography. The data presented are representative of at least three separate experiments.

We next examined the effects of these inhibitors on P-glycoprotein phosphorylation in intact cells. Staurosporine was the most potent, inhibiting both basal and TPA-stimulated phosphorylation (Fig. 6A). Calphostin C was also effective, inhibiting basal phosphorylation by 80% and inhibiting TPA-induced phosphorylation by about 50% (Fig. 6B). As judged by trypan blue exclusion, cells treated with staurosporine and calphostin C retained full viability under these experimental conditions. However, ET-18-OCH_3 at 50 μM, an effective concentration *in vitro* (Fig. 5), was toxic; at 5 μM, with cells maintaining full viability, basal phosphorylation was reduced 30% and TPA-induced phosphorylation was reduced by about 20% (Fig. 6C). Determination of acid-precipitable radioactivity of aliquots of the whole-cell extracts utilized in this experiment demonstrated that the inhibitors did not alter $^{32}P_i$ uptake by the cells.

Effect of inhibitors on vinblastine accumulation. The effects of the PKC inhibitors on vinblastine accumulation in both cell lines, in incubation mixtures where verapamil was omitted, are presented in Table 1. Staurosporine increased vinblastine accumulation in KB-V1 cells by 4-fold while, conversely, slightly decreasing accumulation in KB-3 cells under the same conditions. Preincubation with staurosporine for longer periods resulted in further increases in drug accumulation (up to 10-fold), but this was associated with a progressive loss of cell viability. Calphostin C also increased vinblastine accumulation in KB-V1 cells, by 3-fold, with only a marginal effect on KB-3 cells. The effect of calphostin C on drug accumulation in KB-V1 cells was not increased further by longer periods of preincubation, although viability was maintained. Finally, ET-18-OCH_3 at 5 μM, the highest concentration testable, did not significantly alter vinblastine accumulation levels in either cell line.

TABLE 1

Effects of PKC inhibitors on vinblastine accumulation

Cells were preincubated for 1 hr with inhibitors in serum-free medium and were then incubated for an additional 1 hr in the same medium containing 36 nM [^{3}H] vinblastine without verapamil. Other details are described in Experimental Procedures. Cell viability was >95%. Each value is a mean ± standard error (three experiments).

Inhibitor	Vinblastine accumulation in cells	
	KB-V1	KB-3
	pmol/mg of protein	
None (control)	1.07 ± 0.23	25.7 ± 1.3
Staurosporine (0.5 μM)	4.30 ± 0.15[a]	20.2 ± 1.7
Calphostin C (5 μM)	3.23 ± 0.18[a]	22.1 ± 4.1
ET-18-OCH_3 (5 μM)	1.10 ± 0.25	26.2 ± 2.3

[a] Significantly different from control ($p < 0.005$).

Discussion

Based on comparative one-dimensional phosphopeptide analysis of partially digested P-glycoprotein, we have previously suggested that the same regions are phosphorylated *in vitro* by PKC and in intact KB-V1 cells treated with or without TPA (21). In the present study, we have used the more rigorous approach of two-dimensional tryptic phosphopeptide mapping to confirm that the same three phosphopeptides are obtained from P-glycoprotein, whether phosphorylated in membranes by exogenous PKC or isolated from ^{32}P-labeled intact cells treated with or without TPA or okadaic acid (Fig. 3). In addition, calphostin C (44) and ET-18-OCH_3 (43), two PKC inhibitors that provide specificity by interacting with the regulatory domain of the kinase, inhibited basal phosphorylation of P-glycoprotein in intact cells (Fig. 6). To our knowledge, these results are the first to demonstrate inhibition of P-glycoprotein phosphorylation by PKC-selective inhibitors. The results of Figs. 3 and 6, taken together, argue persuasively that P-glycoprotein is a physiological substrate of PKC. Due to its toxicity at higher concentrations, ET-18-OCH_3 was used at a suboptimal concentration (5 μM) near the IC_{50} (8 μM) for PKC. However, the high degree of inhibition by calphostin C at 5 μM (IC_{50} for PKC = 0.05 μM, IC_{50} for cAMP-dependent protein kinase = 50 μM) (44) implicates PKC as the major enzyme in phosphorylation. A possible contribution from other protein kinases, phosphorylating similar sites, is not ruled out, in view of the greater degree of inhibition by staurosporine versus calphostin C or ET-18-OCH_3 (Fig. 6). Alternatively, this may simply reflect the greater potency or perhaps membrane permeability of staurosporine, compared with the other inhibitors.

Increased phosphorylation of P-glycoprotein in response to TPA has been documented in two other MDR cell lines. In Adriamycin-resistant K562/ADM cells, two major tryptic phosphopeptides of P-glycoprotein in control cells, with an additional two phosphopeptides for TPA-treated cells, were identified (19). These findings suggest that the basal phosphorylation of P-glycoprotein in K562/ADM cells is catalyzed by a protein kinase distinct from PKC. In the human breast cancer (MCF-7)-derived cell lines BC-19/3 and BC-19/15, which are double-transfected with P-glycoprotein and PKC-α plasmids,

two major and three minor tryptic phosphopeptides of P-glycoprotein were identified, with TPA stimulating phosphorylation of the two major preexisting sites (25). Analysis of P-glycoprotein phosphorylation in unstimulated vincristine-resistant HL60 cells has revealed three major phosphorylated tryptic peptides (23). In these studies, as well as our own, it is not presently clear whether individual phosphopeptides represent individual sites of phosphorylation or overlapping peptides containing a common site. Determination of the number and location of phosphorylation sites is important, because the information will provide a basis for analysis of the role of phosphorylation, using methods of site-directed mutagenesis. *In vitro*, P-glycoprotein is phosphorylated by cAMP-dependent protein kinase (31) and by a calcium-independent, phospholipid-dependent protein kinase (32). More detailed analyses, using specific inhibitors and comparing sites of phosphorylation *in vitro* and in intact cells, will be required to determine whether these kinases phosphorylate P-glycoprotein in intact cells. In KB-V1 cells, our results clearly implicate PKC as being chiefly responsible. It will be interesting to determine whether other MDR cell lines also utilize primarily PKC, or other kinases, to phosphorylate P-glycoprotein.

Because the level of phosphorylation of P-glycoprotein depends on the relative activities of both protein kinases and phosphatases, we have begun characterizing reactions catalyzed by the latter enzymes. Our studies have revealed a membrane-associated phosphatase activity that is active in dephosphorylation of P-glycoprotein and sensitive to inhibition by 200 nM okadaic acid (Fig. 1). Of the four major classes of protein phosphatases, only two, PP1 and PP2A, are inhibited by submicromolar or nanomolar concentrations of the toxin (45). The results of Fig. 1 implicate PP1 and/or PP2A in the dephosphorylation of PKC-phosphorylated P-glycoprotein, consistent with the concept that these phosphatases are primarily responsible for the reversal of the actions of PKC (46). The phosphorylation of membrane-bound P-glycoprotein by endogenous PKC (21) and dephosphorylation by PP1/PP2A indicates an association of substrate/kinase/phosphatase in purified membranes vesicles from KB-V1 cells. Other membrane proteins regulated by phosphorylation, for example, phospholamban (47) and calcium-activated potassium channels (48), are similarly associated with their respective regulatory kinase and phosphatase activities.

In contrast to its potent effect *in vitro* (Fig. 1), okadaic acid only modestly increased P-glycoprotein phosphorylation in intact cells (Fig. 2). Preliminary evidence suggests that this may be due to a reduced intracellular concentration of the toxin in drug-resistant cells.[1] In this regard, other PP1/PP2A inhibitors, such as calyculin A (45), may be useful in further characterization of P-glycoprotein dephosphorylation.

The present results confirm and extend our earlier suggestion (33) that PKC-catalyzed phosphorylation stimulates the drug transport activity of P-glycoprotein. Under optimal conditions for increasing phosphorylation of P-glycoprotein, TPA clearly and significantly decreased verapamil-induced (and, to a lesser extent, basal) vinblastine accumulation in drug-resistant cells. In contrast, TPA did not significantly alter levels of vinblastine accumulation in drug-sensitive cells. These results would appear to rule out the possibility of a nonspecific effect of TPA on drug levels and are consistent with its actions being mediated through phosphorylation of P-glycoprotein. The hypothesis that PKC-catalyzed phosphorylation increases the drug-pumping capacity of P-glycoprotein is supported by studies with the inhibitors staurosporine and calphostin C. Thus, under conditions in which these agents inhibit phosphorylation (Fig. 6), there is a concomitant increase in cellular vinblastine accumulation (Table 1). A direct involvement of P-glycoprotein phosphorylation again is likely, because these inhibitors did not increase drug accumulation in KB-3 cells. We note that neither the small but significant decrease in phosphorylation caused by ET-18-OCH_3 (Fig. 6) nor the small but significant increase caused by okadaic acid (Fig. 2) resulted in observable changes in accumulation of vinblastine in KB-V1 cells. It is probable that only substantial increases and decreases in phosphorylation, perhaps inducing conformational alterations in the protein, result in measurable changes in drug transport. Although treatment of KB-V1 cells with staurosporine and calphostin C inhibited phosphorylation and increased drug accumulation, the drug levels achieved were considerably lower than those found in drug-sensitive cells (Table 1). Phosphorylation may thus play a modulatory rather than essential role, perhaps allowing drug-resistant cells to increase the drug-pumping capacity of P-glycoprotein rapidly and efficiently.

Recent studies with the transfectant lines BC-19/15 and BC-19/3 have suggested a qualitative relationship between P-glycoprotein phosphorylation and reduced drug accumulation (25). In addition, consistent with our findings, staurosporine has been found to inhibit P-glycoprotein phosphorylation potently in vincristine-resistant HL60 cells, and this correlates with inhibition of drug efflux, resulting in increased drug retention (23). Other reports have described partial reversal of drug resistance by putative PKC inhibitors (30, 49). However, in those studies, P-glycoprotein phosphorylation was not examined and, therefore, it is not known whether resistance reversal by the inhibitors actually involved inhibition of P-glycoprotein phosphorylation or other mechanisms.

Our studies support a role of PKC in MDR through phosphorylation and modulation of P-glycoprotein activity. Nonetheless, an involvement of PKC in drug resistance independent of P-glycoprotein seems likely. For example, TPA treatment of some drug-sensitive cell lines, lacking P-glycoprotein, can lead to the acquisition of a phenotype resembling MDR (28, 50–52). The mechanisms underlying these observations are not clear but may involve phosphorylation by PKC of other proteins or enzymes implicated in drug resistance, such as glutathione *S*-transferase (53) and topoisomerase II (54). The multiplicity of factors modulated by PKC in drug resistance could potentially be mediated through specific isoforms of the enzyme. In this regard, the presence of PKC-γ in HL60/ADR cells (55) is certainly intriguing.

Acknowledgments

We are grateful to Dr. Michael Gottesman and colleagues at the National Cancer Institute for generously providing the cell lines used in this study. We thank Robert Raynor of this laboratory for kindly providing preparations of PKC. We also thank Stephanie Sanders for her skillful preparation of the manuscript and Dr. Richard Venema for helpful suggestions.

References

1. Pastan, I., and M. M. Gottesman. Multidrug resistance. *Annu. Rev. Med.* **42**:277–286 (1991).
2. Debenham, P. G., N. Kartner, L. Siminovitch, J. R. Riordan, and V. Ling. DNA-mediated transfer of multiple drug resistance and plasma membrane glycoprotein expression. *Mol. Cell. Biol.* **2**:881–889 (1982).
3. Gros, P., Y. B. Neriah, J. M. Croop, and D. E. Housman. Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* (*Lond.*) **323**:728–731 (1986).

4. Shen, D.-W., A. Fojo, I. B. Roninson, J. E. Chin, R. Soffir, I. Pastan, and M. M. Gottesman. Multidrug resistance of DNA-mediated transformants is linked to transfer of the human *mdr1* gene. *Mol. Cell. Biol.* **6**:4039–4044 (1986).
5. Ueda, K., C. Cardarelli, M. M. Gottesman, and I. Pastan. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad. Sci. USA* **84**:3004–3008 (1987).
6. Sugimoto, Y., and T. Tsuruo. DNA-mediated transfer and cloning of a human multidrug-resistant gene of Adriamycin-resistant myelogenous leukemia K562. *Cancer Res.* **47**:2620–2625 (1987).
7. Gros, P., J. Croop, and D. Housman. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell* **47**:371–380 (1986).
8. Chen, C.-J., J. E. Chin, K. Ueda, D. P. Clark, I. Pastan, M. M. Gottesman, and I. B. Roninson. Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**:381–389 (1986).
9. Cornwell, M. M., T. Tsuruo, M. M. Gottesman, and I. Pastan. ATP-binding properties of P-glycoprotein from multidrug-resistant KB cells. *FASEB J.* **1**:51–54 (1987).
10. Hamada, H., and T. Tsuruo. Characterization of the ATPase activity of the M_r 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM cells. *Cancer Res.* **48**:4926–4932 (1988).
11. Cornwell, M. M., M. M. Gottesman, and I. Pastan. Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. *J. Biol. Chem.* **261**:7921–7928 (1986).
12. Kamimoto, Y., Z. Gatmaitan, J. Hsu, and I. M. Arias. The function of Gp170, the multidrug resistance gene product, in rat liver canalicular membrane vesicles. *J. Biol. Chem.* **264**:11693–11698 (1989).
13. Cornwell, M. M., I. Pastan, and M. M. Gottesman. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J. Biol. Chem.* **262**:2166–2170 (1987).
14. Yusa, K., and T. Tsuruo. Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res.* **49**:5002–5006 (1989).
15. Foxwell, B. M. J., A. Mackie, V. Ling, and B. Ryffel. Identification of the multidrug resistance-related P-glycoprotein as a cyclosporine binding protein. *Mol. Pharmacol.* **36**:543–546 (1989).
16. Carlsen, S. A., J. E. Till, and V. Ling. Modulation of drug permeability in Chinese hamster ovary cells: possible role for phosphorylation of surface glycoproteins. *Biochim. Biophys. Acta* **467**:238–250 (1977).
17. Garman, D., L. Albers, and M. S. Center. Identification and characterization of a plasma membrane phosphoprotein which is present in Chinese hamster lung cells resistant to Adriamycin. *Biochem. Pharmacol.* **32**:3633–3637 (1983).
18. Roy, S. N., and S. B. Horwitz. A phosphoglycoprotein associated with taxol resistance in J774.2 cells. *Cancer Res.* **45**:3856–3863 (1985).
19. Hamada, H., K.-I. Hagiwara, T. Nakajima, and T. Tsuruo. Phosphorylation of the M_r 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumor cells: effects of verapamil, trifluoperazine, and phorbol esters. *Cancer Res.* **47**:2860–2865 (1987).
20. Richert, N. D., L. Aldwin, D. Nitecki, M. M. Gottesman, and I. Pastan. Stability and covalent modification of P-glycoprotein in multidrug-resistant KB cells. *Biochemistry* **27**:7607–7613 (1988).
21. Chambers, T. C., E. M. McAvoy, J. W. Jacobs, and G. Eilon. Protein kinase C phosphorylates P-glycoprotein in multidrug resistant human KB carcinoma cells. *J. Biol. Chem.* **265**:7679–7686 (1990).
22. Myers, M. B., L. Rittmann-Grauer, J. P. O'Brien, and A. R. Safa. Characterization of monoclonal antibodies recognizing a M_r 180,000 P-glycoprotein: differential expression of the M_r 170,000 P-glycoprotein in multidrug-resistant human tumor cells. *Cancer Res.* **49**:3209–3214 (1989).
23. Ma, L., D. Marquardt, L. Takemoto, and M. S. Center. Analysis of P-glycoprotein phosphorylation in HL60 cells isolated for resistance to vincristine. *J. Biol. Chem.* **266**:5593–5599 (1991).
24. Schurr, E., M. Raymond, J. C. Bell, and P. Gros. Characterization of the multidrug resistance protein expressed in cell clones stably transfected with the mouse *mdr1* cDNA. *Cancer Res.* **49**:2729–2734 (1989).
25. Yu, G., S. Ahmad, A. Aquino, C. R. Fairchild, J. B. Trepel, S. Ohno, K. Suzuki, T. Tsuruo, K. H. Cowan, and R. I. Glazer. Transfection with protein kinase Cα confers increased multidrug resistance to MCF-7 cells expressing P-glycoprotein. *Cancer Commun.* **3**:181–189 (1991).
26. Posada, J. A., E. M. McKeegan, K. F. Worthington, M. J. Morin, S. Jaken, and T. R. Tritton. Human multidrug resistant KB cells overexpress protein kinase C: involvement in drug resistance. *Cancer Commun.* **1**:285–292 (1989).
27. Palayoor, S. T., J. M. Stein, and W. N. Hait. Inhibition of protein kinase C by antineoplastic agents: implications for drug resistance. *Biochem. Biophys. Res. Commun.* **148**:718–725 (1987).
28. Fine, R. L., J. Patel, and B. A. Chabner. Phorbol esters induce multidrug resistance in human breast cancer cells. *Proc. Natl. Acad. Sci. USA* **85**:582–586 (1988).
29. Aquino, A., K. D. Hartman, M. C. Kuode, S. Grant, K.-P. Huang, C.-H. Niu, and R. I. Glazer. Role of protein kinase C in phosphorylation of vinculin in Adriamycin-resistant HL-60 leukemia cells. *Cancer Res.* **48**:3324–3329 (1988).
30. O'Brian, C. A., D. Fan, N. E. Ward, C. Seid, and I. J. Fidler. Level of protein kinase C activity correlates directly with resistance to Adriamycin in murine fibrosarcoma cells. *FEBS Lett.* **246**:78–82 (1989).
31. Mellado, W., and S. B. Horwitz. Phosphorylation of the multidrug resistance associated glycoprotein. *Biochemistry* **26**:6900–6904 (1987).
32. Staats, J., D. Marquardt, and M. S. Center. Characterization of a membrane-associated protein kinase of multidrug-resistant HL60 cells which phosphorylates P-glycoprotein. *J. Biol. Chem.* **265**:4084–4090 (1990).
33. Chambers, T. C., I. Chalikonda, and G. Eilon. Correlation of protein kinase C translocation, P-glycoprotein phosphorylation and reduced drug accumulation in multidrug resistant human KB cells. *Biochem. Biophys. Res. Commun.* **169**:253–259 (1990).
34. Girard, P. R., G. J. Mazzei, and J. F. Kuo. Immunological quantitation of phospholipid/Ca^{2+}-dependent protein kinase and its fragments. *J. Biol. Chem.* **261**:370–375 (1986).
35. Post, R. L., and A. K. Sen. ^{32}P-labeling of a (Na^++K^+)-ATPase intermediate. *Methods Enzymol.* **10**:773–775 (1967).
36. Shen, D., C. Cardarelli, J. Hwang, M. M. Cornwell, N. Richert, S. Ishii, I. Pastan, and M. M. Gottesman. Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, Adriamycin, or vinblastine show changes in expression of specific proteins. *J. Biol. Chem.* **261**:7762–7770 (1986).
37. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*Lond.*) **227**:680–685 (1970).
38. Gracy, R. W. Two-dimensional thin-layer methods. *Methods Enzymol.* **47**:195–204 (1977).
39. Fojo, A., S.-I. Akiyama, M. M. Gottesman, and I. Pastan. Reduced drug accumulation in multiply drug-resistant human KB carcinoma cell lines. *Cancer Res.* **45**:3002–3007 (1985).
40. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
41. Bialojan, C., and A. Takai. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* **256**:283–290 (1988).
42. Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita. Staurosporine, a potent inhibitor of phospholipid/calcium dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**:397–402 (1986).
43. Helfman, D. M., K. C. Barnes, J. M. Kinkade, Jr., W. R. Vogler, M. Shoji, and J. F. Kuo. Phospholipid-sensitive Ca^{2+}-dependent protein phosphorylation system in various types of leukemic cells from human patients and in human leukemic cell lines HL60 and K562, and its inhibition by alkyllysophospholipid. *Cancer Res.* **43**:2955–2961 (1983).
44. Kobayashi, E., H. Nakano, M. Morimoto, and T. Tamaoki. Calphostin C (UNC-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **159**:548–553 (1989).
45. Cohen, P., C. F. B. Holmes, and Y. Tsukitani. Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* **15**:98–102 (1990).
46. Cohen, P., and P. T. W. Cohen. Protein phosphatases come of age. *J. Biol. Chem.* **264**:21435–21438 (1989).
47. Kranias, E. G. Regulation of calcium transport by protein phosphatase activity associated with cardiac sarcoplasmic reticulum. *J. Biol. Chem.* **260**:11006–11010 (1985).
48. Chung, S., P. H. Reinhart, B. L. Martin, D. Brautigan, and I. B. Levitan. Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. *Science* (*Washington D. C.*) **253**:560–562 (1991).
49. Weisenthal, L. M., Y.-Z. Su, T. E. Duarte, P. L. Dill, and R. A. Nagourney. Perturbation of *in vitro* drug resistance in human lymphatic neoplasms by combinations of putative inhibitors of protein kinase C. *Cancer Treat. Rep.* **71**:1239–1243 (1987).
50. O'Connor, T. W. E. Phorbol ester-induced loss of colchicine ultrasensitivity in chronic lymphocytic leukemia lymphocytes. *Leukemia Res.* **9**:885–895 (1985).
51. Ferguson, P. J., and Y.-C. Cheng. Transient protection of cultured human cells against antitumor agents by 12-*O*-tetradecanoylphorbol-13-acetate. *Cancer Res.* **47**:433–441 (1987).
52. Ido, M., T. Asao, M. Sakurai, M. Inagaki, M. Saito, and H. Hidaka. An inhibitor of protein kinase C, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), inhibits TPA-induced reduction of vincristine uptake from P388 murine leukemic cells. *Leukemia Res.* **10**:1063–1069 (1986).

53. Taniguchi, H., and W. Pyerin. Glutathione *S*-transferase is an *in vitro* substrate of Ca^{++}-phospholipid-dependent protein kinase (protein kinase C). *Biochem. Biophys. Res. Commun.* **162**:903–907 (1989).
54. Sahyoun, N. E., M. Wolf, J. Besterman, T.-S. Hsienh, M. Sander, H. LeVine, K.-J. Chang, and P. Cuatrecasas. Protein kinase C phosphorylates topoisomerase II: topoisomerase activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. *Proc. Natl. Acad. Sci. USA* **83**:1603–1607 (1986).
55. Aquino, A., B. S. Warren, J. Omichinski, K. D. Hartman, and R. I. Glazer. Protein kinase C-γ is present in Adriamycin-resistant HL-60 leukemia cells. *Biochem. Biophys. Res. Commun.* **166**:723–728 (1990).

Send reprint requests to: Dr. Timothy C. Chambers, Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322.