Differential Modulation of P-Glycoprotein Transport by Protein Kinase Inhibition

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ABSTRACT: Previous studies of P-glycoprotein have demonstrated that its function can be modulated by phosphorylation. In the present study, inhibition of protein kinase C with calphostin C or stauroporine or prolonged treatment with the phorbol ester TPA decreased phosphorylation of P-glycoprotein, and impaired transport of vinblastine. Calphostin C also inhibited transport of actinomycin D, vincristine, rhodamine, and azidopine in SW620 Ad300 multidrug-resistant human colon carcinoma cells. Photoaffinity labeling of P-glycoprotein with azidopine was decreased by calphostin C, suggesting that dephosphorylation alters the affinity of P-glycoprotein for its substrates. Impaired transport of rhodamine in normal T lymphocytes treated with staurosporine demonstrates that modulation of P-glycoprotein function is not limited to cells selected for drug resistance *in vitro*. Transport of P-glycoprotein antagonists in SW620 Ad300 cells was also affected by calphostin C. Cyclosporin A transport decreased, while verapamil transport increased. Cyclosporin A in calphostin C-treated cells resulted in additive P-glycoprotein antagonism, while no additive effect could be demonstrated with verapamil, suggesting that the increase in verapamil transport makes it a poorer P-glycoprotein antagonist. These studies suggest that transport by P-glycoprotein is a dynamic process which can be modulated by phosphorylation, and that antagonists may block P-glycoprotein differently in different phosphorylation states.

Studies of P-glycoprotein, which confers multidrug resistance by active chemotherapy drug efflux, have demonstrated transport of a variety of agents (Pastan & Gottesman, 1987). However, these agents, which include dyes and hormones as well as chemotherapeutic agents, appear to be transported differently in different model systems. Differences in cross-resistance patterns emerging after selection of cell lines *in vitro* with different chemotherapeutic agents have been ascribed to differences in other mechanisms of resistance or in one case to a point mutation in P-glycoprotein which changed a glycine at position 185 to a valine (Choi et al., 1988). Likewise, differences in the ability of antagonists to block P-glycoprotein-mediated drug efflux have been ascribed to effects on cellular physiology which occur in addition to competitive inhibition of P-glycoprotein (Ford & Hait, 1990). Recent findings suggest that posttranslational modification of P-glycoprotein can also result in differential transport of various agents. Studies have reported that enhancement of protein kinase C activity by the phorbol ester TPA can increase vinblastine transport in multidrug-resistant cells (Chambers et al., 1990; Fine et al., 1988). Since P-glycoprotein phosphorylation is increased by TPA treatment, these results suggest that alterations in phosphorylation can modulate drug transport and may be responsible for at least some of the differences in cross-resistance patterns. Previous work in our laboratories extended these observations by demonstrating that sodium butyrate treatment of some human colon cancer cell lines impairs P-glycoprotein phosphorylation (Bates et al., 1992). The decrease in phosphorylation is accompanied by decreased transport of vinblastine, Adriamycin, and actinomycin D. However, transport of colchicine is not affected. Thus, the efflux of individual drugs is affected, rather than the absolute transport capability of the protein. To pursue these observations, we evaluated other modulators of P-glycoprotein phosphorylation for their effect on P-glycoprotein transport specificity.

We examined the effect of putative inhibitors of protein kinase C, studying the effects of calphostin C, staurosporine, and long-term TPA treatment. Calphostin C is reported to be a specific inhibitor of protein kinase C, which affects activity by binding to the regulatory domain of PKC (Kobayashi et al., 1989). This agent requires photoactivation for full activity (Bruns et al., 1991). Staurosporine is not specific for protein kinase C, inhibiting its activity through the catalytic domain (Tamaoki et al., 1992). TPA, which activates protein kinase C, also results in down-regulation of the protein kinase C level and activity after continued exposure to the drug (Nishizuka, 1988). We began these studies seeking to determine whether the alterations we observed in P-glycoprotein transport after sodium butyrate treatment, which appeared to be different for different agents, would be present after inhibition of protein kinase C.

EXPERIMENTAL PROCEDURES

Materials. RPMI 1640, Hank's balanced salt solution (HBSS) without phenol red, and improved minimum essential medium (IMEM) without phenol red were obtained from Biofluids, Rockville, MD. Lymphocyte separation medium (LSM) was purchased from Organon Teknika Corp., Durham, NC. Nylon wool fiber was ordered from Polysciences, Inc., Warrington, PA. Rhodamine 123, verapamil, and TPA (phorbol 12-myristate 13-acetate) were products of Sigma Chemical Co., St. Louis, MO. Enlightning was an NEN product. Calphostin C was obtained from Kamiya Biomedical Co., Thousand Oaks, CA. Staurosporine was purchased from Boehringer Mannheim, Indianapolis, IN. Anti-human Leu 9 (CD7) was a product of Becton–Dickinson Immunocytometry Systems, San Jose, CA. Affinity-isolated goat anti-(mouse immunoglobulin R)–phycoerythrin conjugate was ordered from Tago, Inc., Burlingame, CA. The flow cytometer used was a Becton Dickinson FACScan with laser excitation

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of 488 nm. FACScan Research software was used in the acquisition and analysis of data. Antibodies against protein kinase C isoforms were obtained from GIBCO–BRL, Rockville, MD.

Cell Lines. SW620 Ad300 multidrug-resistant cells were derived from parental SW620 human colon carcinoma cells by stepwise selection in Adriamycin (Lai et al., 1991). These cells have been extensively characterized and found to have increased P-glycoprotein as the principal mechanism of resistance. The cells are 76-fold-resistant to Adriamycin compared to the parental cells, and are maintained in 300 ng/mL Adriamycin (Lai et al., 1991). Other cell lines used include the MCF-7 human breast cancer cell line and its drug-resistant subline, MCF-7TH, which was derived by intermittent exposure to Adriamycin and is maintained in 200 ng/mL Adriamycin; the LS 180 Vb20 cell line which was derived by stepwise selection of LS 180 human colon carcinoma cells in vinblastine, and is maintained in 20 ng/mL vinblastine; the HCT-15 human colon carcinoma cell line which, like LS 180 and SW620, was established from a tumor obtained from a patient who had never received chemotherapy; and KB 3-1 human epidermoid cancer cells which are known not to express P-glycoprotein (Herzog et al., 1992; Dexter et al., 1979). All three parental colon cell lines (SW620, LS 180, and HCT-15) express P-glycoprotein, and have been extensively characterized (Lai et al., 1991). Modulation of protein kinase activity was performed by preincubating cells in TPA, calphostin C, or staurosporine in standard medium. Calphostin C-treated cells were exposed to fluorescent light inside the incubator.

Drug Accumulation Assays. Accumulation studies were performed as previously described (Fojo et al., 1985). Cells were plated in six-well dishes 3 days before accumulation studies. Cells were treated with 200 nM calphostin C, 200 nM staurosporine, or 200 nM TPA for 1 h prior to assay, or as indicated in the figures. Cells were rinsed and incubated with assay medium before the start of the incubation. The accumulation assays were performed with 2×10^6 dpm of drug per well, equivalent to 14 nM [^{3}H]vinblastine, 30 nM [^{3}H]actinomycin D, 80 nM [^{3}H]vincristine, 6 nM [^{3}H]-verapamil, 10 nM [^{3}H]azidopine, and 57 nM [^{3}H]cyclosporin A. All modulators which had been added were also present in the assay medium.

Drug efflux studies were performed by incubating cells in 2×10^6 dpm of [^{3}H]vinblastine, [^{3}H]cyclosporin A, [^{3}H]-verapamil, or [^{3}H]actinomycin D per well for 1 h prior to the efflux assay; under these conditions, steady state is not achieved. After a 15-s wash to remove drug-containing medium, sequential exchanges of medium were performed at the indicated time points, and the amount of drug in the medium was determined by scintillation counting.

Metabolic Labeling. Untreated cells or cells pretreated with protein kinase modulators were labeled as previously described (Bates et al., 1992; Richert et al., 1988) with 250 μCi of [^{32}P]orthophosphoric acid (specific activity 6000 Ci/mM) in 4 mL of phosphate-free DMEM without serum for 1 h. TPA was added for the indicated times up to 48 h prior to harvesting the cells; for the 1-h treatment, TPA was added at the start of labeling. The other modulators were added 1 h prior to addition of ^{32}P and continued during the cell labeling. After lysis of cells in RIPA buffer (1% Triton X-100/0.1% $NaDodSO_4$/1% sodium deoxycholate/0.15 M NaCl/20 mM Tris-HCl pH 7.2) with 20 μg/mL aprotinin and centrifugation to remove insoluble material, immunoprecipitation was carried out with a polyclonal antibody raised against recombinant protein fragments (Tanaka et al., 1990); the product was analyzed on a 7.5% SDS–polyacrylamide gel.

Azidopine Labeling. Calphostin C-treated and untreated cells were harvested and resuspended at 2×10^6 cells/50 mL in phosphate-buffered saline (PBS) for labeling with 1 μCi of [^{3}H]azidopine as previously described (Bruggemann et al., 1989). Cells were incubated for 1 h at 37 °C in the dark with gentle shaking. Then the cells were photolabeled with a UV lamp (General Electric F15T8) on ice for 30 min. Extraction and lysis were done in buffer containing 10 mM Tris, pH 7.5, 0.1% Triton X-100, 1 mM DTT, 10 mM $MgSO_4$, and 10 mM $CaCl_2$. Lysis was accomplished by three freeze/thaw cycles and three 1-min cycles in an ultrasonic water bath. The samples were then incubated at 37 °C for 15 min with DNase I. Laemmli buffer was added, and the samples were run overnight on a 5%/7.5% SDS–polyacrylamide gel. After gel enhancement with Enlightning (NEN), the gel was dried for 2 h under vacuum and prepared for autoradiography.

T Cell Separation. Peripheral blood from healthy volunteers was collected in heparinized syringes. Blood samples were diluted 1:1 with PBS, and mononuclear cells were obtained by centrifugation over Ficoll. The mononuclear cells were collected from the plasma–LSM interface and were washed twice with RPMI + 5% FBS to remove platelets, plasma, and LSM. T cells were isolated by running the obtained lymphocytes over a nylon–wool column. The column was prepared by packing "fluffed" nylon–wool fiber in a 60-mL syringe (4 g/column) and washing with RPMI + 10% FBS. The mononuclear cell suspension was incubated with the column for 1 h at 37 °C and 5% CO_2, and the T cells were eluted with 25 mL of prewarmed medium. The collected T cells were washed twice in RPMI + 10% FBS and aliquoted onto plates for experimentation.

T Cell Immunostaining. T cell immunostaining was performed as previously described (Coon et al., 1991) to document the effectiveness of the nylon–wool column. A total of 5×10^6 cells were resuspended in PBA (PBS with 1% albumin and 0.1% sodium azide) and incubated with a 1:500 dilution of anti-Leu 9/CD7 antibody. After being washed, the cells were resuspended in 150 μL of anti-(mouse immunoglobulin R)–phycoerythrin conjugate diluted 1:80 with PBA. After the mixture was washed, the cells were resuspended in PBA for FACS analysis, which demonstrated the success of the T cell column and required only limited exclusion of non-T cells by appropriate gating.

Rhodamine 123 Accumulation. T cells were aliquoted into six-well dishes immediately before the assay, while cell lines were plated in 100-mm dishes 48 h prior to assay. Following treatment with or without modulators, rhodamine 123 (1 μg/mL) was added to the plates, and incubated in the dark at 37 °C in 5% CO_2 for 30 min. Cells that were allowed to efflux were placed in rhodamine-free IMEM (without phenol red) + 10% FBS. The efflux was carried out at 37 °C for the designated time periods. At the end of incubation or efflux, cells were trypsinized if attached, washed in ice-cold HBSS (without phenol red), placed in HBSS + 10% FBS on wet ice, and kept in the dark until FACS analysis. Samples were analyzed on a FACScan flow cytometer (Becton–Dickinson) equipped with a 488-nm argon laser. The green fluorescence of rhodamine 123 was collected after a 530-nm band-pass filter (hLi). Samples were gated on forward scatter vs side scatter to exclude debris and clumps. A minimum of 5000 events were collected on each sample.

Protein Kinase C Isoform Immunoblotting. Pretreated and untreated cells were harvested in protein kinase C buffer (2

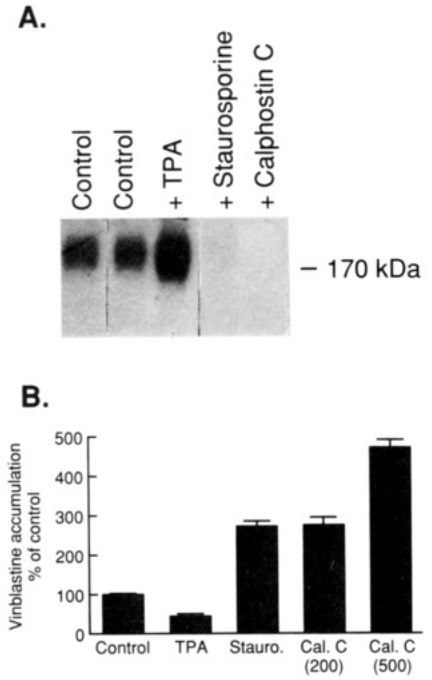

FIGURE 1: Effect of protein kinase C inhibitors on P-glycoprotein phosphorylation and vinblastine accumulation in SW620 Ad300 cells. (A) Cells were labeled in 4 mL of phosphate-free DMEM with 250 μCi of [^{32}P]orthophosphate for 1 h prior to harvesting and immunoprecipitation. (B) Vinblastine accumulations were performed simultaneously for 1 h. Staurosporine- and calphostin C-treated cells were pretreated with 200 nM staurosporine or calphostin C for 1 h before the assays. Both drugs were also present during the assay, and 200 nM TPA was added at the start of the labeling or of the vinblastine accumulation. A signal could be detected on longer exposures in the cells treated with staurosporine or calphostin C.

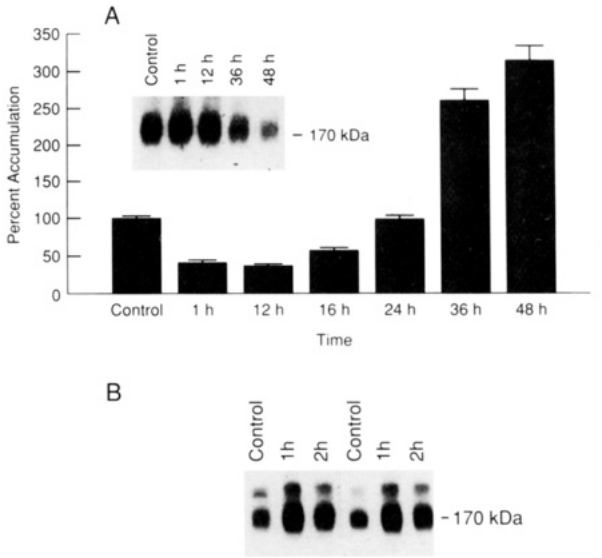

FIGURE 2: Effect of TPA on P-glycoprotein phosphorylation and vinblastine accumulation in SW620 Ad300 cells. (A) Untreated cells or cells pretreated with 200 nM TPA for the duration indicated were labeled for 1 h as in Figure 1, with TPA treatment continuing during labeling. Following harvesting, immunoprecipitation and electrophoresis were carried out. Simultaneous studies of [^{3}H]-vinblastine accumulation were carried out. (B) Repeat phosphorylation studies with TPA treatment for 1 and 2 h in cells sparsely plated (left panel) and densely plated (right panel).

mM EDTA, 0.5 mM EGTA, 5 mM DTT, 25 mM Tris, 50 μg/mL PMSF, 5 μg/mL leupeptin, and 50 μg/mL aprotinin) (Leach et al., 1988). After sonication, the disrupted cells were centrifuged at 1000g to remove nuclei; the samples were then centrifuged at 100000g for 30 min. The supernatant, containing the cytosol, was removed, and the membrane pellet was resuspended in protein kinase C buffer. After protein quantification (BioRad), equal amounts (75 μg) of cytosolic and membrane protein were loaded with Laemmli buffer on a 5/7.5% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose in Towbin transfer buffer. The nitrocellulose containing the protein was blocked for 6 h with 1% BSA in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20). The blot was then incubated overnight in TBST with 2 μg/mL of the appropriate protein kinase C antibody. After the blot was washed 3 times in TBST, detection was obtained using ^{125}I-labeled secondary antibody in TBST for 30 min. After three washes, the blot was prepared for autoradiography.

RESULTS

To examine the effects of known inhibitors of protein kinase C on the phosphorylation and function of P-glycoprotein, we utilized a multidrug-resistant subline designated SW620 Ad300, which was derived from SW620 human colon cancer cells by exposure to Adriamycin. In the experiments shown in Figure 1, cells plated 48 h prior to assay were pretreated with 200 nM staurosporine or calphostin C for 1 h before the addition of [^{32}P]orthophosphate. Metabolic labeling was carried out at 37 °C for an additional 1 h in the presence of the protein kinase modulators (200 nM TPA was added at the start of labeling). Accumulation of [^{3}H]vinblastine was measured simultaneously in cells treated under the same conditions. As shown in panel A, while acute treatment with TPA increased P-glycoprotein (Pgp) phosphorylation, a marked decrease was observed after treatment with staurosporine and calphostin C. The increased phosphorylation observed following a 1-h TPA treatment was associated with a decrease in vinblastine accumulation to 30% of that in untreated cells (panel B). In contrast, with both inhibitors, an increase in vinblastine accumulation of 250–400% was observed. Comparable results were obtained when protein kinase C activity was inhibited by long-term TPA treatment, as shown in Figure 2. Panel A shows the results of Pgp phosphorylation and vinblastine accumulation in SW620 Ad300 cells treated with TPA. In panel A, the early increase in phosphorylation resulting from the addition of TPA is less dramatic than in either Figure 1 or panel B of Figure 2, in part because a longer exposure is shown but also as a result of experimental variability. Again, the increased phosphorylation after TPA treatment is accompanied by a decrease in vinblastine accumulation presumably due to increased drug efflux. With continued TPA exposure, a decrease in phosphorylation is visible at both 36 and 48 h of treatment with TPA, and this decrease is associated with an increase in vinblastine accumulation. Cell counts obtained in these experiments demonstrated mild growth inhibition, with cell counts in treated cells 80% of that in untreated cells. However, as shown in panel B, these results were independent of cell density, with similar increases observed in cells in the logarithmic phase of growth or in confluent cells.

Previous studies demonstrated that impairment of vinblastine transport does not occur in every cell line treated with sodium butyrate (Mickley et al., 1989). Thus, to determine whether inhibition of protein kinase C would consistently result in increased vinblastine accumulation, four additional cell lines expressing P-glycoprotein were treated with calphostin C. These four cell lines include two multidrug-resistant cell lines selected *in vitro* with vinblastine (LS 180 Vb20) and Adriamycin (MCF-7TH) and two unselected colon cancer cell lines (HCT-15 and SW620). For comparison, results

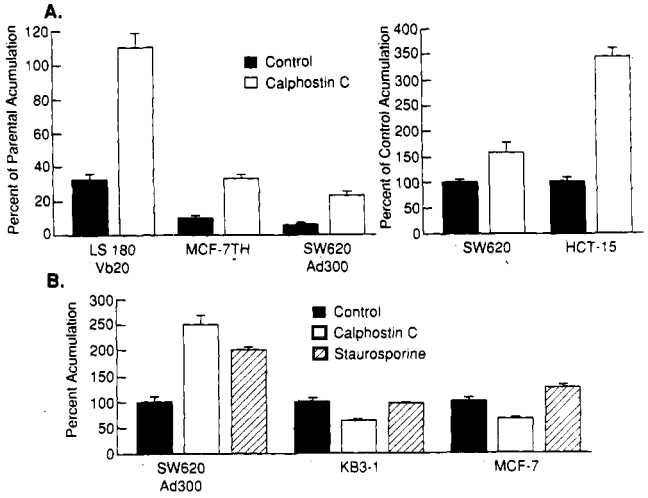

FIGURE 3: Effect of calphostin C on [^{3}H]vinblastine accumulation in seven human cancer cell lines. Cells were plated in six-well dishes 1 day prior to the assay. Pretreatment with 200 nM calphostin C began 1 h before the assay. Accumulation of [^{3}H]vinblastine for 1 h in the presence or absence of calphostin C was performed for the cell lines shown. (Panel A) Results in five cell lines expressing P-glycoprotein are shown. The results with the three drug-selected sublines on the left are expressed relative to the [^{3}H]vinblastine accumulation of their respective parental cells which were arbitrarily assigned a value of 100%. The increase following calphostin C treatment is similar for these three cell lines although the percent of parental accumulation varies. Control (untreated) cells in the unselected parental cell lines on the right were also assigned a value of 100%. (Panel B) Results in two parental cell lines without P-glycoprotein expression are compared to results with SW620 Ad300 cells.

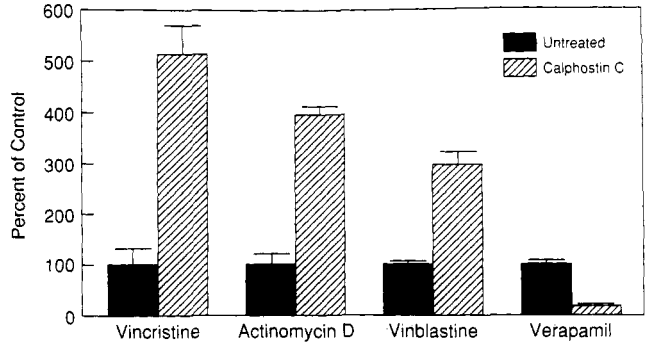

FIGURE 4: Effect of calphostin C on drug accumulation in SW620 Ad300 cells. Cells were plated in six-well dishes the day before the assay and pretreated with or without 200 nM calphostin C for 1 h before measuring drug accumulation in the presence or absence of calphostin C. Vincristine and vinblastine accumulations were performed for 1 h while actinomycin D and verapamil accumulations were carried out for 15 min.

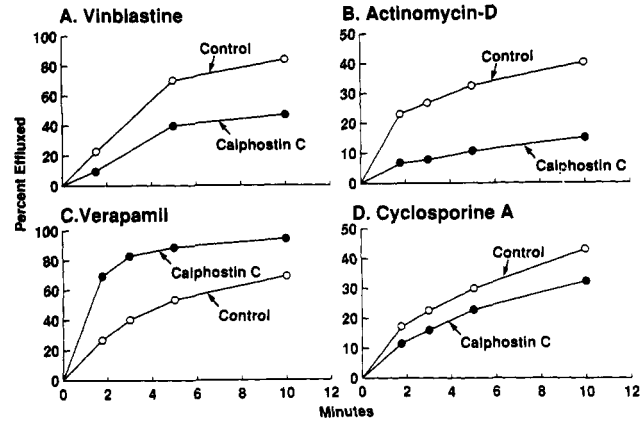

FIGURE 5: Effect of calphostin C on drug efflux in SW620 Ad300 cells. Control cells or cells pretreated with 200 nM calphostin C for 1 h were loaded with ^{3}H-labeled drug for 1 h in the presence or absence of 200 nM calphostin C. Efflux of drug was then measured over 10 min, and is expressed as the percent effluxed, where 100% is the total amount of drug present inside the cells at the end of the drug accumulation. The latter was determined by adding the amount of drug effluxed to that remaining inside the cells at the conclusion of the efflux period. Each point is the mean of triplicate experiments. Error bars are not shown since they are contained inside the symbols.

with the SW620 Ad300 cells shown in Figures 1 and 2 are presented. The results with the selected cell lines are presented as the percent of parental accumulation. Accumulation in parental cells was measured simultaneously and assigned a value of 100%. The three multidrug-resistant sublines have high levels of P-glycoprotein and accumulate [^{3}H]vinblastine between 8 and 34% of parental levels. As shown in Figure 3, panel A, treatment of LS 180 Vb20 cells with calphostin C increased [^{3}H]vinblastine accumulation to a level similar to that of parental cells, while MCF-7TH cells responded to calphostin C to the same degree as SW620 Ad300 cells. SW620 cells have low levels of P-glycoprotein which confer a small degree of drug resistance as demonstrated by an increase in vincristine and actinomycin D toxicity after treatment with verapamil (Herzog et al., 1992). HCT-15 cells have higher levels of P-glycoprotein and demonstrate a typical multidrug-resistant phenotype, although the cells have never been selected for resistance (Lai et al., 1991). As shown on the right in panel A, both of these unselected cell lines demonstrated an increase in vinblastine accumulation after treatment with 200 nM calphostin C. In panel B, KB3-1 and MCF-7 cells, which are known not to express P-glycoprotein (Dexter et al., 1979), were treated with protein kinase C inhibitors to exclude the possibility that the result observed in Figure 1 was due to a nonspecific effect on the cell membrane. As can be seen, although both calphostin C and staurosporine increased accumulation in SW620 Ad300 cells, in the cell lines without Pgp, these agents either had no effect or resulted in a small decrease in vinblastine accumulation. These results suggest that P-glycoprotein phosphorylation mediated at least in part by protein kinase C plays an integral role in the function of P-glycoprotein.

To examine the breadth of agents affected by calphostin C, we evaluated in SW620 Ad300 cells the accumulation of other agents known to be transported by P-glycoprotein: daunorubicin, actinomycin D, cyclosporin A, vincristine, and verapamil. With the exception of verapamil, treatment with calphostin C increased the accumulation of the other agents in SW620 Ad300 cells. As shown in Figure 4, [^{3}H]vincristine and [^{3}H]actinomycin D accumulation increased to a level 400–500% that of untreated cells. Results with [^{3}H]vinblastine are shown for comparison. Cyclosporin A and daunorubicin demonstrated a comparable increase in accumulation (not shown). In contrast, [^{3}H]verapamil accumulation was decreased to 20% of control. Although the amount of drug in untreated cells varies for each agent, all are assigned a value of 100% in the figure.

Since accumulation could be affected by changes in drug influx, studies of drug efflux were performed to confirm that differences in efflux could account for the observed differences in accumulation. As shown in Figure 5, a decrease in efflux for vinblastine, actinomycin D, and cyclosporin A was observed after treatment with calphostin C. Considering the total amount of drug present in cells after the loading period to be 100%, the charts graph the amount of tritiated drug appearing in the media at the time points examined. Only verapamil demonstrated an increase in drug efflux. To be certain that

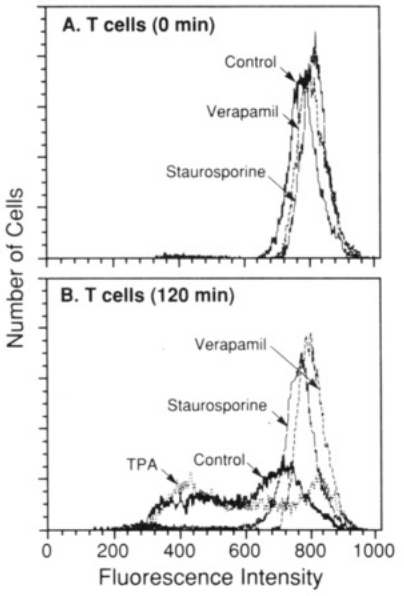

FIGURE 6: Effect of verapamil, staurosporine, and TPA on rhodamine 123 accumulation in T cells obtained from healthy volunteers. Panel A demonstrates the results at the end of a 1-h incubation with 1 μg/mL rhodamine 123. Panel B presents the data after 2 h of efflux, which magnifies the differences. Cells were pretreated for 1 h with 200 nM staurosporine, and all drugs were present during the initial accumulation as well as the 2 h of efflux. The x axis depicts fluorescence intensity in channels. A shift of 77 channels represents a 2-fold difference in accumulation.

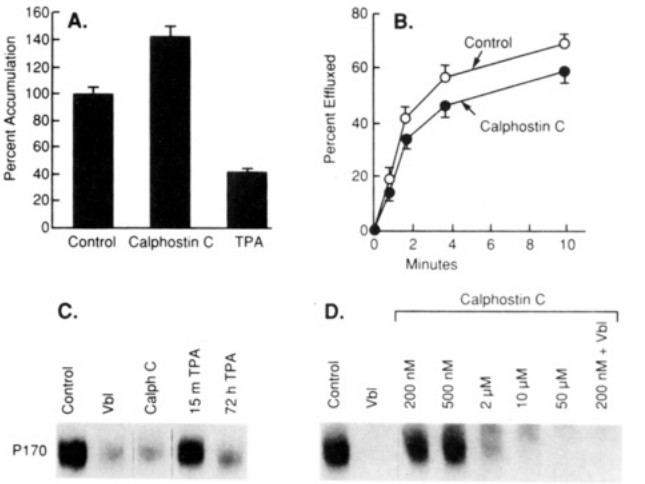

FIGURE 7: Effect of calphostin C on azidopine accumulation, efflux, and photoaffinity labeling in SW620 Ad300 cells. In panels A, B, and C, calphostin C-treated cells were pretreated with 200 nM calphostin C or 200 nM TPA. (Panel A) The [^{3}H]azidopine accumulation after calphostin or TPA was performed for 15 min at 37 °C. (B) Efflux studies were performed on cells pretreated with 200 nM calphostin C, with continued treatment through the loading period. (C) The photolabeling experiment was performed in the presence of 200 nM calphostin C. Azidopine accumulation increased by 50% while efflux over 10 min decreased from 65 to 55%. This was accompanied by a decrease in photoaffinity labeling. (D) Evaluation of direct calphostin competition for affinity labeling. Membranes were prepared and aliquoted for assay in the presence of azidopine and increasing concentrations of calphostin C. Membranes were exposed to UV light for 15 min without preincubation.

verapamil was not in itself causing an increase in efflux despite the addition of only nanomolar quantities in the drug accumulation and efflux studies, [^{3}H]vinblastine accumulation was performed in the presence of 20 and 200 nM verapamil in untreated and in calphostin C-treated cells. These studies demonstrated no effect of 6, 20, and 200 nM verapamil on [^{3}H]vinblastine accumulation. These concentrations exceed those used in the verapamil accumulation study but are not able to block the high levels of P-glycoprotein present in SW620 Ad300 cells.

Since *in vitro* model systems may have artifactual elevations in kinase levels, and this increase may be magnified in cells selected *in vitro* with chemotherapy agents, we sought to determine whether similar results would be obtained in noncultured cells. Normal human T lymphocytes obtained by venipuncture from normal volunteers were separated by a Ficoll–Hypaque density gradient, and treated with 200 nM TPA, calphostin C, or staurosporine. It has been previously reported that drug efflux possibly mediated by P-glycoprotein can be demonstrated in T lymphocytes (Coon et al., 1991; McGown et al., 1991). Rhodamine 123 was chosen for the efflux studies because it appears to be a sensitive indicator of P-glycoprotein activity when assayed by FACS (Efferth et al., 1989; Chaudhary & Roninson, 1991; unpublished results). Results with calphostin C-treated T cells were not interpretable because of cell shrinkage, and hence only staurosporine could be evaluated. Panel A of Figure 6 demonstrates a small increase in rhodamine accumulation after treatment with either verapamil or staurosporine. These results are magnified in panel B, which demonstrates a marked diminution in rhodamine accumulation in control cells after allowing 120 min for rhodamine efflux. Both verapamil and staurosporine appear to prevent rhodamine efflux and maintain a high level of rhodamine in the cells after the efflux period. An effect of TPA was not observed on the initial accumulation (not shown) and was difficult to interpret after a 120-min efflux, although the histograms suggest an overall decrease in rhodamine levels (TPA result shown only in panel B). Although a TPA effect was difficult to discern, concanavalin A stimulation of T cells appeared to increase rhodamine efflux (data not shown). Previous investigators reported that concanavalin A activation of lymphocytes results in activation of protein kinase C (Kvanta et al., 1991).

P-glycoprotein-mediated transport is thought to begin with the binding of drug to P-glycoprotein. To determine the effect of dephosphorylation of P-glycoprotein on drug binding, azidopine labeling was performed. As shown in Figure 7, panel A, calphostin C treatment increased [^{3}H]azidopine accumulation just as was observed for vinblastine, vincristine, actinomycin D, and cyclosporin A. Azidopine accumulation was increased from 100% to 150%. Following treatment with TPA, the accumulation of azidopine was decreased to 28% of control, comparable to the result observed with vinblastine (panel A). The increase in azidopine accumulation was associated with a decrease in efflux after 10 min, from 65% to 55% (panel B). Incubation of cell membranes with [^{3}H]-azidopine followed by exposure to UV light for 30 min labeled a 170-kDa protein, shown in previous studies to be P-glycoprotein (panel C) (Safa et al., 1987; Cornwell et al., 1986). The specificity of labeling was confirmed by competition with vinblastine, which inhibited azidopine labeling. Pretreatment with 200 nM calphostin C for 1 h resulted in a decrease in azidopine labeling. Similar results were obtained with azidopine labeling of intact cells, despite the increase in azidopine present in the cells. These observations suggest that the increase in azidopine accumulation is a result of a decrease in the affinity of P-glycoprotein for azidopine. Pretreatment of cells for 72 h with TPA also resulted in a decrease in azidopine labeling. In contrast, a short-term exposure of cells to TPA (15 min) resulted in no change in azidopine binding, despite the increase in phosphorylation observed in Figures 1 and 2. To exclude the possibility that the decrease in azidopine binding after calphostin C was due

to direct competition of azidopine binding by calphostin C, labeling studies were performed for 15 min in the presence of increasing concentrations of calphostin C without pretreatment. Azidopine labeling was not affected by 200 nM calphostin C, the concentration used in these experiments, nor by incubation with 500 nM calphostin C. A decrease in labeling was observed at higher concentrations, suggesting the possibility that calphostin C, a natural product, may compete azidopine labeling. However, an equally likely possibility is that the higher concentrations affect phosphorylation even in a 15-min incubation. Similar results were obtained when a 15-min vinblastine accumulation in SW620 Ad300 cells was performed in the presence of increasing concentrations of calphostin C. Accumulation was not increased with 200 nM and only minimally with 500 nM calphostin C. Higher concentrations increased drug accumulation (vinblastine accumulations not shown).

Since treatment with calphostin C altered the transport of the P-glycoprotein antagonists verapamil and cyclosporin A in different (opposite) ways, the ability of each to antagonize P-glycoprotein-mediated drug transport was examined in untreated cells and after treatment of cells with calphostin C. Similar results were obtained when rhodamine or vinblastine accumulation and efflux studies were performed with and without the P-glycoprotein antagonists after a 1-h treatment with 200 nM calphostin C. The levels of rhodamine after a 1-h accumulation are shown in Figure 8A. In SW620 Ad 300 cells, rhodamine accumulation increased after treatment with calphostin C compared to untreated cells. In untreated cells, incubation of rhodamine with 10 μg/mL verapamil increased rhodamine levels. Comparable results were obtained with 3 μg/mL cyclosporin A. However, when cells were examined after rhodamine efflux, differences between untreated and calphostin C-treated cells were observed. In untreated cells, 10 μg/mL verapamil was a slightly better antagonist than 3 μg/mL cyclosporin A as can be seen after 10 min of efflux (verapamil in panel B compared to cyclosporin A in panel C). However, following calphostin C pretreatment, rhodamine levels after 10 min of efflux indicated that antagonism by 10 μg/mL verapamil was less effective than 3 μg/mL cyclosporin A (panels B and C of Figure 8). Similar results were observed after 20 and 30 min of efflux (data not shown). The addition of cyclosporin A to calphostin C-pretreated cells resulted in at least an additive effect on rhodamine retention. These results suggest that the enhanced transport of verapamil observed after treatment with calphostin C impairs its performance as a P-glycoprotein antagonist. Results with [^{3}H]vinblastine were similar to the rhodamine results (data not shown).

Although studies to date have not conclusively proven which kinases phosphorylate P-glycoprotein and which residues are modified, the present data are consistent with the hypothesis that protein kinase C plays an important role. Protein kinase C may act alone or with other kinases to regulate P-glycoprotein function. The role of protein kinase C in P-glycoprotein phosphorylation and the effect of calphostin C on protein kinase C were examined in SW620 Ad300 cells by immunoblotting using antibodies to six protein kinase C isoforms: α, β, γ, ϵ, δ and ζ. The α and ζ isoforms were readily detected, and antibodies against these were utilized to evaluate protein kinase C level in cell lysates separated by ultracentrifugation. This approach allows for a discrimination between the activated form of protein kinase C, found in the membrane fraction (P), and the inactive form, found in the cytosol (S). Immunoblot analysis of SW620 Ad300 cells and of SW620 Ad300 cells

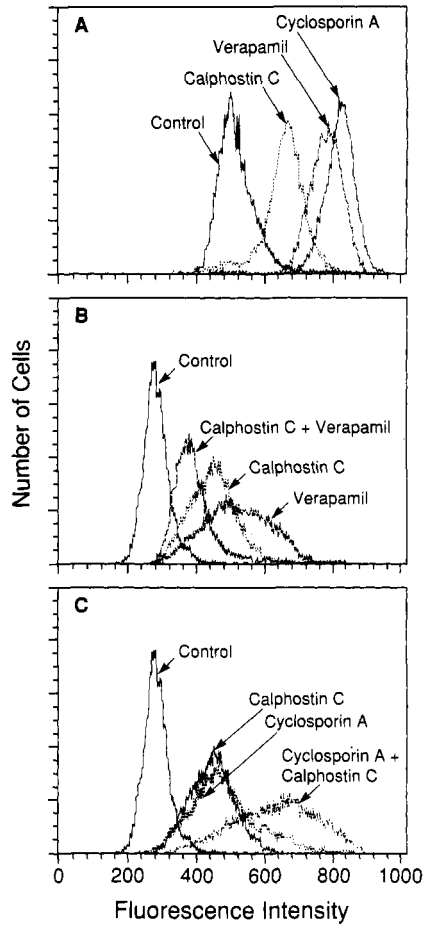

FIGURE 8: Effect of calphostin C on the antagonism of rhodamine 123 by cyclosporin A and verapamil in SW620 Ad300 cells. Untreated control cells or cells pretreated with 200 nM calphostin C for 1 h were compared. Cells were loaded with 1 μg/mL rhodamine 123 for 30 min in the absence of any agent (control) or in the presence of 200 nM calphostin C, 3 μg/mL cyclosporin A, 10 μg/mL verapamil, or a combination of the two agents. Rhodamine fluorescence at the end of the 30-min period of accumulation is shown in panel A. Panels B and C show the results after an additional 10-min efflux period in which cells were incubated under the same conditions utilized during the 30-min loading period, but in the absence of rhodamine. The effect of the antagonists in preventing rhodamine efflux is shown in both untreated and calphostin C-treated cells.

after treatment with TPA and/or calphostin C is shown in Figure 9. The upper panel demonstrates the effect of TPA on the α and ζ isoforms. Membrane-bound protein kinase C α is difficult to detect in untreated cells without activation by TPA. Treatment with TPA results in translocation of protein kinase C α to the membrane fraction, followed by a slow decrease in the total level of protein kinase C in both fractions over the 72-h period examined. In contrast, protein kinase C ζ is detected in the membrane fraction of control cells before TPA, and is largely unaffected by TPA. Calphostin C has no significant effect on protein kinase C α, but affects the distribution of protein kinase C ζ. The level of protein kinase C ζ in the membrane fraction is decreased 3-fold by treatment with calphostin C whether or not TPA is added prior to harvest of the cells. These results suggest that more than one protein kinase C isoform may be involved in phosphorylation of P-glycoprotein.

DISCUSSION

The studies presented here extend earlier observations of alterations in P-glycoprotein function after treatment of multidrug-resistant cells with agents which interfere with

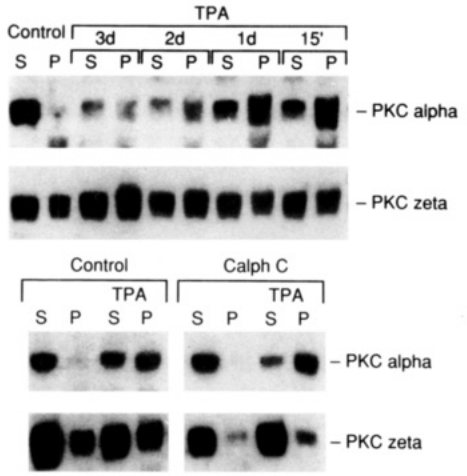

FIGURE 9: Effect of protein kinase C antagonists on the distribution of the α and ζ isoenzymes of protein kinase C in SW620 Ad300 cells. Control cells, cells treated with TPA for the indicated times, or cells treated with 200 nM calphostin C for 1 h were harvested and fractionated into a cytosol (S) fraction and a membrane fraction (P). Following electrophoresis and transfer to nitrocellulose, immunoblotting was performed with polyclonal antibodies specific for the two isoenzymes detected in SW620 Ad300 cells. The effects of TPA on protein kinase C α and of calphostin C on protein kinase C ζ are shown in the upper and lower panels, respectively.

P-glycoprotein phosphorylation. Inhibition of protein kinase C with calphostin C of staurosporine or long-term TPA treatment decreased the phosphorylation of P-glycoprotein and modulated drug accumulation. Increased vinblastine accumulation resulted from impaired P-glycoprotein-mediated drug transport, as demonstrated by a decrease in drug efflux. Multiple compounds which were affected like vinblastine included vincristine, actinomycin D, daunorubicin, rhodamine, azidopine, and the P-glycoprotein antagonist cyclosporin A. However, verapamil transport was increased by treatment with calphostin C. This increased transport was associated with decreased blocking by verapamil, suggesting that enhancing transport impairs the ability to antagonize P-glycoprotein. The mechanism by which P-glycoprotein phosphorylation modulates transport appears to be by affecting drug binding, as demonstrated by azidopine labeling studies. Although proof that protein kinase isoenzyme ζ is directly responsible for phosphorylation of P-glycoprotein is not provided, treatment of cells with calphostin C decreased the level of this isozyme in the membrane fraction. This observation at least confirms the activity of calphostin C on protein kinase C in our model.

Phosphorylation of P-glycoprotein and its effect on function have been previously studied. The work of Ling, Center, and Tsuruo demonstrated that P-glycoprotein was phosphorylated (Center, 1983; Carlsen et al., 1977; Hamada et al., 1987). Tsuruo provided evidence that TPA, verapamil, and trifluoperazine could modulate P-glycoprotein phosphorylation (Hamada et al., 1987). Subsequently, Fine and Chambers demonstrated increased drug resistance after treatment with the phorbol ester TPA (Fine et al., 1988; Chambers et al., 1990). Chambers observed that this increase in drug resistance was mediated by decreased vinblastine accumulation, and was associated with an increase in P-glycoprotein phosphorylation. Other studies associated inhibition of P-glycoprotein phosphorylation by staurosporine with decreased drug efflux (Ma et al., 1991). Our laboratories confirmed increased P-glycoprotein phosphorylation and enhanced P-glycoprotein-mediated vinblastine efflux after TPA treatment (Bates et al., 1992). These studies also demonstrated that sodium butyrate treatment of parental SW620 cells and its multidrug-resistant sublines resulted in decreased P-glycoprotein phosphorylation associated with impaired vinblastine efflux and increased vinblastine accumulation. Although previous studies had suggested a generalized impairment of P-glycoprotein function after dephosphorylation, the sodium butyrate experiments demonstrated that the interference with function could be specific. Colchicine transport was preserved after sodium butyrate treatment, while vinblastine, actinomycin D, and Adriamycin transport was impaired. These studies led to those presented here which also suggest that the effect of phosphorylation and dephosphorylation on P-glycoprotein function varies with individual agents. This can be explained by modulation in the affinity of P-glycoprotein for the agents. Support for the idea that altered affinity is responsible for the modulation in P-glycoprotein transport is provided by the experiments demonstrating decreased azidopine binding after calphostin C. Interestingly, azidopine binding was observed after sodium butyrate treatment of SW620 cells; however, the degree of binding was difficult to assess since sodium butyrate treatment of SW620 cells increased P-glycoprotein levels 25-fold.

If the state of phosphorylation does, in fact, modulate the affinity of P-glycoprotein for various substrates, and in turn their transport, one could also predict differential effects on the ability of P-glycoprotein antagonists to block transport. According to this prediction, some antagonists would be more effective against phosphorylated P-glycoprotein and others against the dephosphorylated protein. If P-glycoprotein-mediated drug efflux is a dynamic process characterized by ongoing phosphorylation and dephosphorylation, then a combination of antagonists may be more effective in achieving maximum blockade of P-glycoprotein-mediated drug efflux. This possibility is consistent with previous observations of synergy with combinations of antagonists, including verapamil and cyclosporin (Hu et al., 1990) and quinine and verapamil (Lehnert et al., 1991). The experiments demonstrating a differential effect of calphostin C on P-glycoprotein antagonism by verapamil and cyclosporin are consistent with this. For rhodamine, impaired verapamil antagonism was suggested by the decreased accumulation, indicating increased efflux, observed after pretreatment with calphostin C compared to untreated controls. In contrast, the inhibition of rhodamine efflux by cyclosporin A was at least additive to the effect of the calphostin C. Thus, antagonism by verapamil was impaired after calphostin C treatment while competition by cyclosporin A was retained and possibly enhanced. With rhodamine, these differences were magnified after allowing time for efflux. Similar observations were made with tritiated vinblastine, although with this drug differences were observed both after the initial accumulation period and in efflux experiments (vinblastine results not shown). These results, together with the demonstration of increased cyclosporin A accumulation and decreased verapamil retention after calphostin C, suggest that the increased rate of transport after calphostin C treatment decreases the ability of verapamil to antagonize the transport of both vinblastine and rhodamine.

While these and previous studies suggest that protein kinase C plays a major role in P-glycoprotein phosphorylation and function, evidence is accumulating that other kinases may also modulate P-glycoprotein phosphorylation (Mellado & Horwitz, 1987; Staats et al., 1990). The possibility of both multiple sites of P-glycoprotein phosphorylation and several kinases is consistent with similar observations in other transmembrane domain proteins including the β-adrenergic

and rhodopsin receptors (Sibley et al., 1987). For P-glycoprotein, the evidence of multiple kinases includes the phosphorylation *in vitro* by protein kinase A, and evidence of novel kinases in multidrug-resistant HL60 cells and in KBV-1 cells (Staats et al., 1990; Irene Abraham, personal communication). Indirect evidence from our studies includes the observation that sodium butyrate treatment results in dephosphorylation of P-glycoprotein, without affecting protein kinase C level or activity, and that TPA can overcome the effect of sodium butyrate (Bates et al., 1992; unpublished observations). In the present study, treatment with TPA for 48–72 h was able to down-regulate protein kinase C α, but not protein kinase C ζ, in agreement with observations in other laboratories (Ways et al., 1992; Ono et al., 1989). Calphostin C, on the other hand, affected protein kinase C ζ and not α. This suggests that at least two isoforms of protein kinase C may be involved in P-glycoprotein phosphorylation. Protein kinase C α is ubiquitous and has been shown to phosphorylate numerous proteins. A specific role of protein kinase C ζ has not yet been identified, but its presence has been demonstrated in a broad range of tissues, including liver and kidney, which express P-glycoprotein (Wetsel et al., 1992; Nishizuka, 1988). PKC ζ has been studied after phorbol ester treatment. No evidence of translocation after acute TPA, and no evidence of down-regulation after long-term TPA, has been obtained (Gschwendt et al., 1992; Ways et al., 1992). Our results in Figure 9 are compatible with these findings. Further, calphostin C could not prevent TPA-induced translocation of PKC α. Interestingly, apparent high levels of expression of PKC ζ were noted in the membrane fraction in our cells without TPA treatment, also seen in the immunoblots of Ways and co-workers (Ways et al., 1992). In view of the apparent down-regulation of PKC ζ in the membrane fraction after calphostin C treatment, it is interesting to speculate that this localization represents endogenous activation, a subject of ongoing investigation. Finally, these data only provide evidence for PKC α and ζ involvement in P-glycoprotein phosphorylation, but do not prove it.

The complexity of the results with the protein kinase isoforms is paralleled by the effects of the protein kinase C modulators on P-glycoprotein function. Calphostin C increases vinblastine and azidopine accumulation, while TPA reduces accumulation. In contrast, verapamil transport is increased by both TPA and calphostin C. Thus, hyperphosphorylation does not necessarily result in the opposite effect of dephosphorylation. This may be explained if different sites are hyperphosphorylated by TPA than are dephosphorylated by calphostin C.

A concern in the study of multidrug resistance is that the *in vitro* models used have high levels of P-glycoprotein, are derived by continuous drug selection, and are usually rapidly growing cells which are continuously having nutrients replenished. These conditions are not representative of most malignancies, which have lower levels of P-glycoprotein, are exposed intermittently to chemotherapy, have much slower doubling rates, and often are poorly vascularized. Except for vascularization, the same is true of normal cells not exposed to chemotherapy. To validate the results in the various *in vitro* models, we chose to study P-glycoprotein function in normal human T cells. These cells have low levels of P-glycoprotein, are not dividing, and in healthy donors have never been exposed to chemotherapy. Previous reports have suggested that T cells demonstrate P-glycoprotein-mediated drug efflux (Coon et al., 1991; McGown et al., 1991). We were able to confirm these findings and to demonstrate that one inhibitor of protein kinase C, staurosporine, could interfere with rhodamine efflux. This suggests that P-glycoprotein in normal T cells is also influenced by phosphorylation. Whether protein kinase C is responsible remains to be determined since staurosporine is not specific for protein kinase C. An effect of TPA was suggested, but not conclusively demonstrated. Concanavalin A, which is reported to activate protein kinase C in T cells (Kvanta et al., 1991), did appear to result in enhanced rhodamine efflux (data not shown). These observations suggest that P-glycoproteins in both normal and selected cells are a dynamic pump, whose activity is modulated at least in part by protein kinase C. Adding to the complexity is the observation of increased P-glycoprotein expression after TPA treatment in normal T cells (Gupta et al., 1992).

The possibility that P-glycoprotein is phosphorylated in normal cells leads to the speculation that, like other membrane receptors, phosphorylation of P-glycoprotein in normal cells may result from binding of "ligand". This phosphorylation could then lead to transport. In malignant and drug-resistant cells, with an array of protein kinases present at higher levels (Posada et al., 1989), there may be constitutive phosphorylation of P-glycoprotein, resulting in transport which is more efficient and less tightly regulated than in normal cells. Pursuit of these studies could result in the design of P-glycoprotein antagonists which enhance drug accumulation in cancer more than in normal tissue. Alternatively, protein kinase inhibitors suitable for clinical use could be expected to increase drug accumulation in cancer cells.

In summary, the studies presented here demonstrate that P-glycoprotein-mediated drug efflux is modulated by phosphorylation both in malignant and in normal cells. For azidopine, this modulation has been demonstrated to be a consequence of decreased binding, suggesting a mechanism by which phosphorylation affects P-glycoprotein function. Modulation of phosphorylation affects both drug transport and the ability of P-glycoprotein antagonists to interfere with transport. In the case of verapamil, dephosphorylation increased verapamil transport, rendering it a weaker antagonist. It is hoped that these studies may further the understanding of P-glycoprotein function and the role of phosphorylation and that this may be exploitable in the treatment of clinical drug resistance.

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