Biochemical Pharmacology, Vol. 58, pp. 1723–1733, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00288-9

Mechanism of Inhibition of P-glycoprotein-Mediated Drug Transport by Protein Kinase C Blockers

Ariel F. Castro, Julie K. Horton,†‡ Carlos G. Vanoye* and Guillermo A. Altenberg*§*

*Department of Physiology and Biophysics, and †Sealy Center for Molecular Sciences, University of Texas Medical Branch, Galveston, TX 77555, U.S.A.

ABSTRACT. P-glycoprotein is a membrane ATPase that transports drugs out of cells and confers resistance to a variety of chemically unrelated drugs (multidrug resistance). P-glycoprotein is phosphorylated by protein kinase C (PKC), and PKC blockers reduce P-glycoprotein phosphorylation and increase drug accumulation. These observations suggest that phosphorylation of P-glycoprotein stimulates drug transport. However, there is evidence that PKC inhibitors directly interact with P-glycoprotein, and therefore the mechanism of their effects on P-glycoprotein-mediated drug transport and the possible role of phosphorylation in the regulation of P-glycoprotein function remain unclear. In the present work, we studied the effects of different kinds of PKC inhibitors on drug transport in cells expressing wild-type human P-glycoprotein and a PKC phosphorylation-defective mutant. We demonstrated that PKC blockers inhibit drug transport by mechanisms independent of P-glycoprotein phosphorylation. Inhibition by the blockers occurs by (i) direct competition with transported drugs for binding to P-glycoprotein, and (ii) indirect inhibition through a pathway that involves PKC inhibition, but is independent of P-glycoprotein phosphorylation. The effects of the blockers on P-glycoprotein phosphorylation do not seem to play an important role, but the PKC-signaling pathway regulates P-glycoprotein-mediated drug transport. BIOCHEM PHARMACOL **58**;11:1723–1733, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. MDR1; phosphorylation; chelerythrine; multidrug resistance; safingol; ABC proteins

Chemotherapy of cancer often fails when cells are intrinsically resistant or develop resistance to chemically unrelated agents. Overexpression of P-glycoprotein (MDR1‖ in humans) can cause such multidrug resistance and has been implicated in the resistance of tumors to chemotherapy [1]. P-glycoprotein is a membrane ATPase that transports drugs out of the cells, decreasing the intracellular concentration of cytotoxic agents and causing multidrug resistance [1, 2]. Studies on the regulation of MDR1-associated drug transport could have important implications for designing protocols to overcome multidrug resistance in cancer.

MDR1 undergoes specific phosphorylation by several kinases [3–5], and PKC inhibitors such as staurosporine, chelerythrine, and calphostin C decrease P-glycoprotein phosphorylation and increase drug accumulation [4, 6–9]. Stimulation of PKC by phorbol esters has opposite effects, i.e. increases P-glycoprotein phosphorylation and multidrug resistance [4, 9, 10]. These observations suggest that phosphorylation of P-glycoprotein accelerates P-glycoprotein-mediated drug transport. However, photoaffinity-labeling studies using [^{3}H]azidopine as a specific P-glycoprotein photolabel, as well as P-glycoprotein binding studies in membrane vesicles, have shown that PKC inhibitors interact directly with P-glycoprotein [11–13]. Hence, the effect of PKC inhibitors on drug transport by P-glycoprotein could be due, at least in part, to direct competition with transported drugs for binding to P-glycoprotein. Hence, the mechanism of inhibition of P-glycoprotein-associated drug transport by PKC blockers and the role of phosphorylation by PKC in the regulation of P-glycoprotein remain unclear.

In the present work, our aim was to elucidate the mechanisms by which PKC blockers inhibit drug transport by P-glycoprotein. To achieve this goal, we evaluated the effects of different kinds of PKC blockers on drug transport, and compared the effects of the blockers in cells expressing wild-type MDR1 and a PKC phosphorylation-defective mutant. Our results show that PKC blockers affect drug transport both (i) independently of the PKC pathway, and (ii) through a signaling pathway that involves PKC inhibition, but is unrelated to direct phosphorylation of P-glycoprotein.

‡ Present address: Laboratory of Structural Biology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

§ Corresponding author: Guillermo Altenberg, M.D., Ph.D., Department of Physiology and Biophysics, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0641. Tel. (409) 772-3026; FAX (409) 772-3381; E-mail: Galtenbe@utmb.edu

‖ *Abbreviations*: MDR1, human multidrug resistance protein; PKC, protein kinase C; PKA, protein kinase A; BCECF-AM, 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester; R123, rhodamine 123; F_{R123}, R123 fluorescence; and *k*, rate constant for R123 efflux.

Received 11 January 1999; accepted 21 May 1999.

MATERIALS AND METHODS

Cell Lines

We employed human breast cancer cell lines MCF-7 (drug-sensitive) and BC19/3 (MCF-7 cells transfected with human *MDR1* cDNA and selected with doxorubicin), and mouse fibroblast cell lines [see Refs. 14 and 15 for additional information on the cell lines]. The fibroblast cells were BALB-3T3 (drug-sensitive), BALB-3T3–1000 (high level MDR1 expression, BALB-3T3 cells transfected with *MDR1* cDNA and selected with vinblastine), BALB-MDR1 (BALB-3T3 cells transfected with wild-type *MDR1* cDNA), and BALB-MDR1–3SA (BALB-3T3 transfected with a PKC phosphorylation-defective *MDR1* cDNA). BALB-MDR1 and BALB-MDR1–3SA cells were not selected with chemotherapeutic agents and express P-glycoprotein at low levels. In the mutant MDR1, the serines phosphorylated by PKC were substituted by alanines. Cell culture conditions were as described [14, 15].

PKC Inhibitors

Staurosporine (0.45 mM in DMSO), chelerythrine (5 mM in water), and calphostin C (1.3 mM in DMSO) were purchased from the Alexis Corp. The corresponding solvent was used as the control. When safingol (10 mM in ethanol, Calbiochem) was used, fatty acid-free BSA was added to the solutions at a final concentration of 0.1 mM to reduce toxic effects [16]. BSA was present in the control experiments and slightly increased calcein accumulation (<20%).

Engineering and Expression of Wild Type and PKC Phosphorylation-Defective MDR1

Full-length *MDR1* cDNA cloned into the *Xba*I site of pGEM-3z (Promega, all other cloning sites were removed) was purchased from the American Type Culture Collection (ATCC 65705), and modified by the addition of six histidine residues at the C-terminus end. The histidine tag could be used for protein detection and partial purification. MDR1 with a histidine tag transports drugs and confers multidrug resistance (see Results). Details on the addition of the histidine tag will be described elsewhere. To engineer the PKC phosphorylation-defective MDR1, a 1.6-kb fragment of the human *MDR1* cDNA (cut with *Eco*RI and *Kpn*I) was cloned into the p-Alter-1 vector (Promega) digested with the same enzymes. Site-directed mutagenesis was carried out in three steps with the Altered Sites II *in vitro* mutagenesis system (Promega). The mutagenic oligonucleotides were: 5′-TTCAAGATCCGCGCTAATAAGAA-3′ (Ser [661] to Ala), 5′-AAGAAAAAGAGCCACTCGT-AGG-3′ (Ser^{667} to Ala), and 5′-ACTCGTAGAGCGG-TCCGTGGATC-3′ (Ser^{671} to Ala). Bases changes are underlined. The Ser^{671} to Ala mutagenic oligonucleotide includes one additional base mutation (silent) that adds a *Bsr*BI site for easy screening. The primary screening of the Ser^{661} to Ala and Ser^{667} to Ala mutants was done with *Hin*P1 I (site added) and *Dpn*I (site removed), respectively. Double-stranded DNA sequencing was performed to confirm the presence of the mutations. An *Acc*I fragment from the 1.6 kb mutant *MDR1* cDNA was isolated and used to replace the wild-type *Acc*I fragment of MDR1. Finally, the wild-type and mutant *MDR1* cDNAs were cloned into the *Xba*I site of the mammalian expression vector pLK-444M. This vector was constructed by adding the *Hin*dIII to *Bam*HI multiple cloning site of pGEM-4z (Promega) to pLK-444 (gift of Dr. P. Melera, *Sal*I, *Hin*dIII and *Bam*HI cloning sites) [17]. The unique restriction sites in the multiple cloning site of pLK-444M are *Hin*dIII, *Sph*I, *Pst*I, *Hin*cII, *Acc*I, *Xba*I, and *Bam*HI. The vector pLK-444M has a β-actin promoter and contains the neomycin phosphotransferase gene that was used for selection of stable transfectants with the antibiotic geneticin (G418), which does not induce multidrug resistance [18]. Cell transfections were performed using Lipofectin (Gibco, BRL), and stable clones were obtained as previously described, without exposure of the cells to chemotherapeutic agents [18]. BALB-3T3 cells were used as hosts because they are suitable for stable expression of P-glycoprotein and do not express measurable MDR1 or MRP [15, 18, 19].

MDR1 Expression by Western Blot Analysis and Flow Cytometry

Western blots of crude membranes were performed using standard techniques, as previously described [15, 19]. For flow cytometry, cells were scraped and pelleted by centrifugation at 1000 *g* for 10 min. Then the cells were washed twice by centrifugation at 400 *g* for 5 min in PBS (68 mM NaCl, 75 mM sodium phosphate, pH 7.4) with 2% fetal bovine serum (FBS). The cells resuspended in PBS with FBS were incubated with 10 μg of 4E3-FITC, an anti-MDR1 monoclonal antibody (Signet), for 30 min in the dark at room temperature. Then the cells were washed twice as described above and finally resuspended in 0.5 mL of PBS with 2% FBS. Cells were stored at 4°, in the dark, and analyzed within 1 hr. Analysis was performed on a FACSscan (Becton Dickinson) at the Flow Cytometry Facility of the University of Texas Medical Branch. Confocal immunofluorescence studies showed an absence of intracellular fluorescence labeling.

MDR1 Phosphorylation

The PKC phosphorylation reaction (200 μL volume) contained: 20 mM Tris–HCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 250 mM sucrose, 10 μM [γ-^{32}P]ATP (3 x 10^4 cpm/pmol), 50 μg/mL of phosphatidylserine, 10 μg/mL of diacylglycerol, 500 μg of crude membranes, and 1 unit of PKC, pH 7.5. The reaction mixture was incubated for 30 min at room temperature, and was stopped by the addition of 10 mM EDTA. The suspension was centrifuged for 30 min at 260,000 *g*, and the pellet was washed twice with 100 mM

Na_2CO_3, pH ~12, to remove peripheral membrane proteins. The pellet was resuspended in 1 mL of ice-cold PBSTDS. PBSTDS is a solubilization buffer consisting of PBS containing 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM sodium fluoride, 200 μM sodium orthovanadate, 5 mM EDTA, 2 μg/mL of aprotinin, 5 μg/mL of leupeptin, 0.1 μg/mL of pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4. After a 30-min incubation, the sample was centrifuged at 10,000 *g* for 20 min, and the resulting supernatant was mixed with 30 μL of protein A-agarose (Pierce) and incubated for 30 min with continuous gentle mixing on a tube rotator. Then the sample was centrifuged at 10,000 *g* for 5 min. The protein A-agarose incubation step was performed to deplete the sample from proteins that bind to protein A-agarose non-specifically. The C219 antibody (10 μg) was added to the supernatant, vortex mixed, and incubated for 2 hr. The suspension was then centrifuged at 10,000 *g* for 5 min, and 1 mL PBSTDS was added to the pellet and mixed gently. The washing of the agarose beads was repeated three times. Finally, 100 μL of Laemmli electrophoresis sample buffer was added, and after a 1-hr incubation at 37° the sample was centrifuged at 10,000 *g* for 5 min and the supernatant was electrophoresed (7% SDS–PAGE). The phosphorylation was estimated by autoradiography at −70° after a 15-day film exposure using intensifying screens.

Unidirectional Efflux of R123 and Steady-State Accumulation of R123 and Vinblastine

R123 efflux was determined at 37° by quantitative fluorescence microscopy, as described [14, 18, 20]. The control HCO_3^-/CO_2-buffered solution had the following composition: 115 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.5 mM sodium phosphate, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 7.8 mM glucose, pH 7.40 to7.45 once equilibrated with 5% CO_2/95% O_2. The decay of intracellular F_{R123} was measured from 100 to 150 cells. F_{R123} decays with a time course that is well fit by a single exponential, and the rate constant for F_{R123} decay (k) can be determined from:

$$F_{R123} = F_0 + F_{R123}(0)e^{-kt} \quad (1)$$

where F_0 is the background fluorescence, and $F_{R123}(0)$ is F_{R123} at $t = 0$.

For determinations of steady-state levels of R123, cells were exposed to 10 μM R123 for 1 hr and R123 content was determined by fluorometry, as previously described [15, 20]. For steady-state vinblastine accumulation studies, cells were loaded in a HEPES-buffered solution (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 7.8 mM glucose, and 5 mM HEPES, pH 7.4), with [^{3}H]vinblastine (55 nM, 0.5 μCi/mL, Moravek) for 2 hr, at room temperature. Radioactivity was quantitated by scintillation counting and cell number by nuclei count [21].

Calcein Accumulation

Calcein accumulation was measured as described [22, 23]. Cells were trypsinized, resuspended in HEPES-buffered solution, and placed in a cuvette (10^5/mL) on a spectrofluorometer (CMT1, Spex Industries). The increase in fluorescence produced by the addition of calcein-AM (Molecular Probes) was determined at room temperature, during continuous stirring. Excitation and emission wavelengths were 495 and 515 nm, respectively.

Drug Resistance Assays

Cytotoxicity was determined by growth inhibition assays. Cells were seeded at a density of 20,000 cells/well in six-well tissue culture dishes, and after 24 hr the medium was replaced by medium containing serial dilutions of cytotoxic agents. Cells were counted after 4 days of continuous exposure to the agents (5–6 doubling times) by a nuclei lysis procedure [24].

Photoaffinity Labeling of MDR1 with [^{3}H]Azidopine

Photoaffinity labeling of MDR1 with [^{3}H]azidopine was carried out essentially as described [15, 21]. For *in vivo* studies, we used cells resuspended in HEPES-buffered Ringer's solution (5×10^5 cells/assay). For *in vitro* studies, we used crude membranes prepared as described [15]. Cells or membranes were preincubated for 30 min in the absence or presence of drugs (e.g. vinblastine, staurosporine, chelerythrine). Then [^{3}H]azidopine (55 Ci/mmol, Amersham) was added to a final concentration of 0.5 μM, and the incubation was allowed to proceed for 60 min in the dark, at room temperature. The samples were UV irradiated, and then cells were sonicated and centrifuged at 270,000 *g* for 30 min. The pellets were resuspended in SDS buffer and electrophoresed (7% SDS–PAGE). Gels were fixed in 7% acetic acid/25% methanol, incubated for 30 min with AMPLIFY® (Amersham), and dried. Fluorographs were obtained at −70° (3- to 4-day exposure).

Statistics and Data Presentation

Data were analyzed using Student's *t*-test for paired or unpaired data, as appropriate. $P < 0.05$ was considered significant. Data are presented as means ± SEM. When only single examples are shown, the traces are representative of at least three separate experiments. Gel experiments were repeated a minimum of two times. Control data from the parental BALB-3T3 cells are presented throughout, but data from a G418-resistant cell line transfected with pLK-444M were identical.

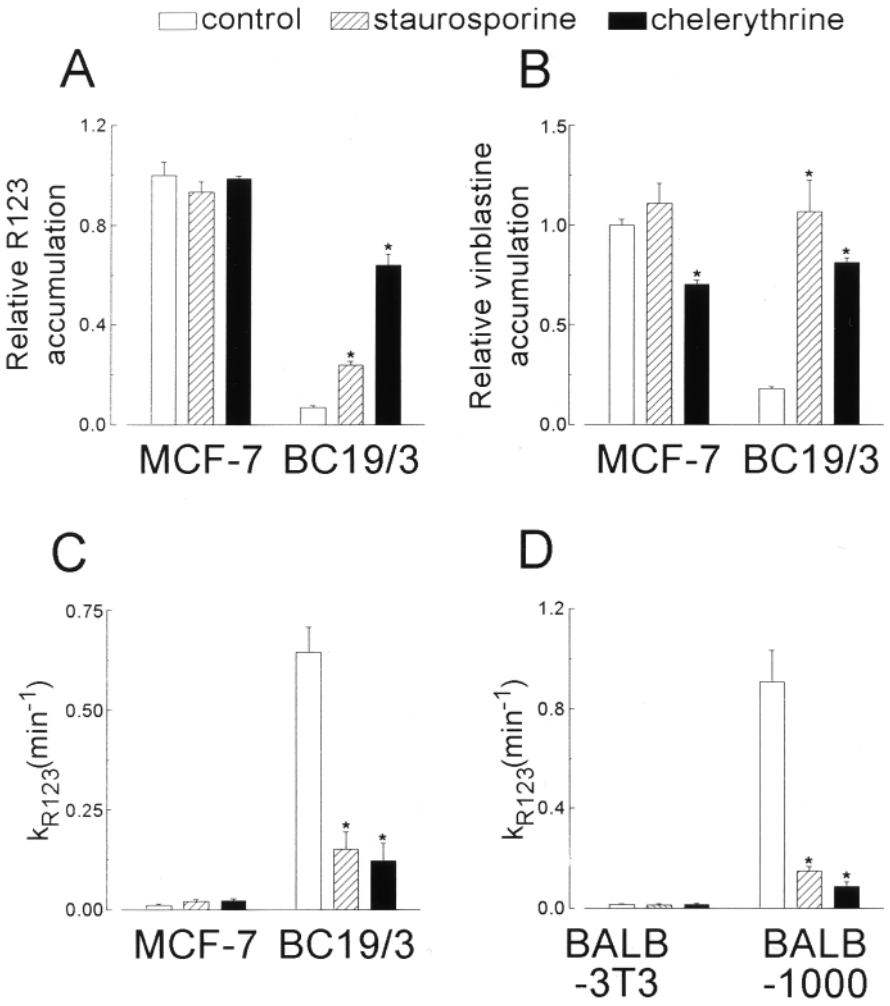

FIG. 1. Effects of staurosporine and chelerythrine on drug accumulation and efflux. (A) Effects on R123 accumulation in breast cancer cells. Average control R123 accumulation in MCF-7 cells was 11 nmol/10^6 cells. (B) Effects on vinblastine accumulation in breast cancer cells. Average control vinblastine accumulation in MCF-7 cells was 46 pmol/10^6 cells. (C) Effects on R123 efflux in breast cancer cells. (D) Effects on R123 efflux in mouse fibroblasts. For drug accumulation studies, the cells were loaded for 1 hr with either 10 μM R123 or 55 nM vinblastine in the absence or presence of staurosporine (6 μM) or chelerythrine (100 μM). Drug accumulation results were normalized to the average value in MCF-7 cells. Data are means ± SEM of 4–12 experiments. Key: (*) indicates statistical significance ($P < 0.05$) compared with the corresponding control, in the absence of PKC inhibitors.

RESULTS

Effects of PKC Inhibitors on Drug Accumulation and Efflux

Initially, we studied the effects of PKC blockers on MDR1-associated drug transport in breast cancer cell lines. The BC19/3 cells display multidrug resistance associated with the expression of MDR1 [14, 15]. In agreement with previous observations [6–10], we found that staurosporine and the more specific PKC blocker chelerythrine increased steady-state levels of R123 and vinblastine only in the MDR1-expressing cells (Fig. 1, A and B). Calphostin C, another specific PKC blocker, also increased R123 accumulation in BC19/3 cells (data not shown). In MCF-7 cells, chelerythrine reduced vinblastine accumulation, an effect opposite to that in BC19/3 cells (Fig. 1B). However, chelerythrine had no effect on R123 accumulation (Fig. 1A). The effect of chelerythrine on vinblastine accumulation in MCF-7 cells was not studied further. Although the results suggested that inhibition of PKC affects MDR1-associated drug efflux, we performed direct R123 efflux studies to confirm that the effect of the PKC blockers on R123 accumulation was due to a decrease in efflux. As expected, staurosporine and chelerythrine decreased the rate of R123 efflux (Fig. 1C). The effects of PKC blockers were not cell-specific because they also reduced R123 efflux in the multidrug-resistant MDR1-expressing mouse fibroblasts BALB-1000 (Fig. 1D).

Next, we determined the time course of the effect of PKC blockers on R123 efflux in MDR1-expressing cells. We reasoned that since the rate of phosphorylation/dephosphorylation of MDR1 is approximately 30 min [25], an effect due to inhibition of PKC would take minutes to develop and to reverse upon removal of the blocker. On the other hand, the onset and the reversal of an effect due to direct competition for transport will likely be faster. Figure 2 illustrates a typical experiment in BC19/3 cells loaded with R123 and initially washed without drugs. After a few minutes, staurosporine was added to the superfusate, and a rapid inhibition was observed. A reversal of the inhibition clearly was apparent after *ca.* 2 min of staurosporine removal. On average, staurosporine reduced the rate constant for R123 efflux to 15 ± 3% of control within 2 min ($N = 4$, $P < 0.05$). The reversal of the block after 5 min was partial (to 36 ± 10% of control). A faster onset than reversal is expected from a competition direct block, but a dual effect consisting in a fast competition and a slower

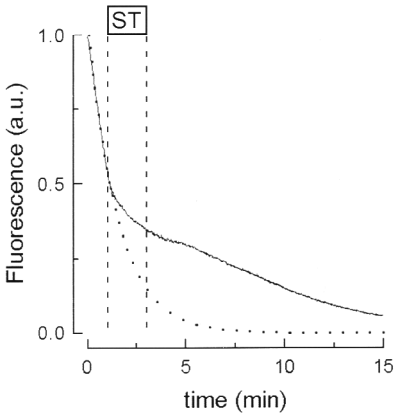

FIG. 2. Rapid inhibition of R123 efflux by staurosporine in BC19/3 cells. The cells were loaded with 10 μM R123 in HCO_3^-/CO_2-Ringer's solution for 1 hr, at 37°, and then were superfused on the stage of an inverted microscope with R123-free solution. Staurosporine (6 μM) was present in the superfusate during the time indicated (ST). The dotted line is the best fit of Equation 1 to the data before the addition of staurosporine. Fluorescence was measured at 525 to 550 nm, with excitation at 495 nm. This trace is representative of six similar experiments.

effect due to inhibition of MDR1 phosphorylation is also possible.

Functional Expression of MDR1 and a PKC Phosphorylation-Defective Mutant

The results presented above strongly suggest that the effects of PKC blockers on MDR1-associated drug transport are due, at least in part, to direct competition for transport. To demonstrate that the blocker effects are independent of their effects on MDR1 phosphorylation, we decided to study the effects of PKC blockers in cells expressing a PKC phosphorylation-defective mutant. A full-length MDR1 in which Ser^{661}, Ser^{667}, and Ser^{671} were substituted with alanine residues (MDR1–3SA) was engineered. The serines substituted in MDR1–3SA were chosen based on the identification of MDR1 residues phosphorylated by PKC [3, 4, 26]. The parental MDR1-devoid BALB-3T3 cells were transfected with either wild-type *MDR1* cDNA or PKC phosphorylation-defective mutant *MDR1–3SA* cDNA, and stable clones were obtained. We expanded and studied several transfected cell lines expressing MDR1 and MDR1–3SA. Figure 3A illustrates the relationship between the rate constant for R123 efflux and membrane P-glycoprotein expression (see Fig. 3B). The cell lines were not selected with chemotherapeutic agents and display relatively low levels of expression of MDR1 or MDR1–3SA. The R123 efflux data indicate that both proteins were functional, that there was a linear relationship between MDR1 expression and R123 transport rate, and that the R123 transport capability of MDR1–3SA did not seem to differ from that of MDR1 (Fig. 3A). Since two of the cell lines expressing either MDR1 (BALB-MDR1) or MDR1–3SA (BALB-MDR1–3SA) had the same expression level (boxed data in

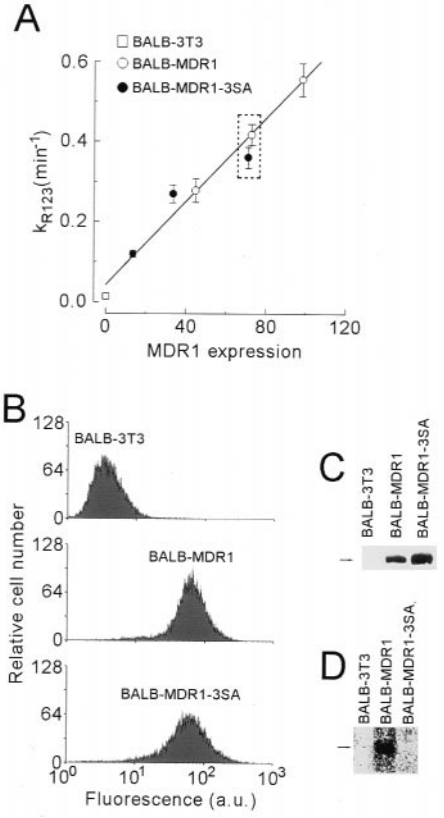

FIG. 3. Expression and phosphorylation by PKC of MDR1 and the PKC phosphorylation-defective mutant MDR1–3SA. (A) Rate constants for R123 efflux as a function of plasma membrane expression of P-glycoprotein. The efflux experiments (means ± SEM, N = 5–10) were performed essentially as shown in Fig. 2. Plasma membrane expression of MDR1 and MDR1–3SA (N = 3–6) was determined by flow cytometry as shown in panel B and expression was normalized to the value in the cell line with the highest expression. (B) Flow cytometry. Cells were labeled with the anti-MDR1 antibody 4E3 conjugated to FITC. Shown is a representative experiment, which was performed five times. (C) Western blot. Membranes (100 μg) from BALB-3T3, BALB-MDR1, and BALB-MDR1–3SA were probed with the anti-P-glycoprotein monoclonal antibody C219. (D) Phosphorylation by PKC. Membranes (500 μg) were phosphorylated by PKC in the presence of [γ-^{32}P]ATP, immunoprecipitated with C219, subjected to SDS–PAGE, and autoradiographed.

Fig. 3A), these cell lines were chosen for subsequent studies. The rate of efflux of R123 in BALB-MDR1 and BALB-MDR1–3SA cells was undistinguishable and > 50-fold faster than that of BALB-3T3 cells. The calculated rate constants for R123 efflux were 0.008 ± 0.004 min^{-1} in BALB-3T3 cells (N = 4), 0.464 ± 0.057 min^{-1} in BALB-MDR1 cells (N = 5), and 0.480 ± 0.045 min^{-1} in BALB-MDR1–3SA cells (N = 5). The flow-cytometry results using the external epitope anti-MDR1 antibody 4E3 show that expression of MDR1 and MDR1–3SA at the plasma membrane was not different (Fig. 3B). Panels C and D of Fig. 3 show that MDR1–3SA phosphorylation by PKC was not detectable, while Fig. 4 illustrates that the level of multidrug resistance in BALB-MDR1 and BALB-MDR1–

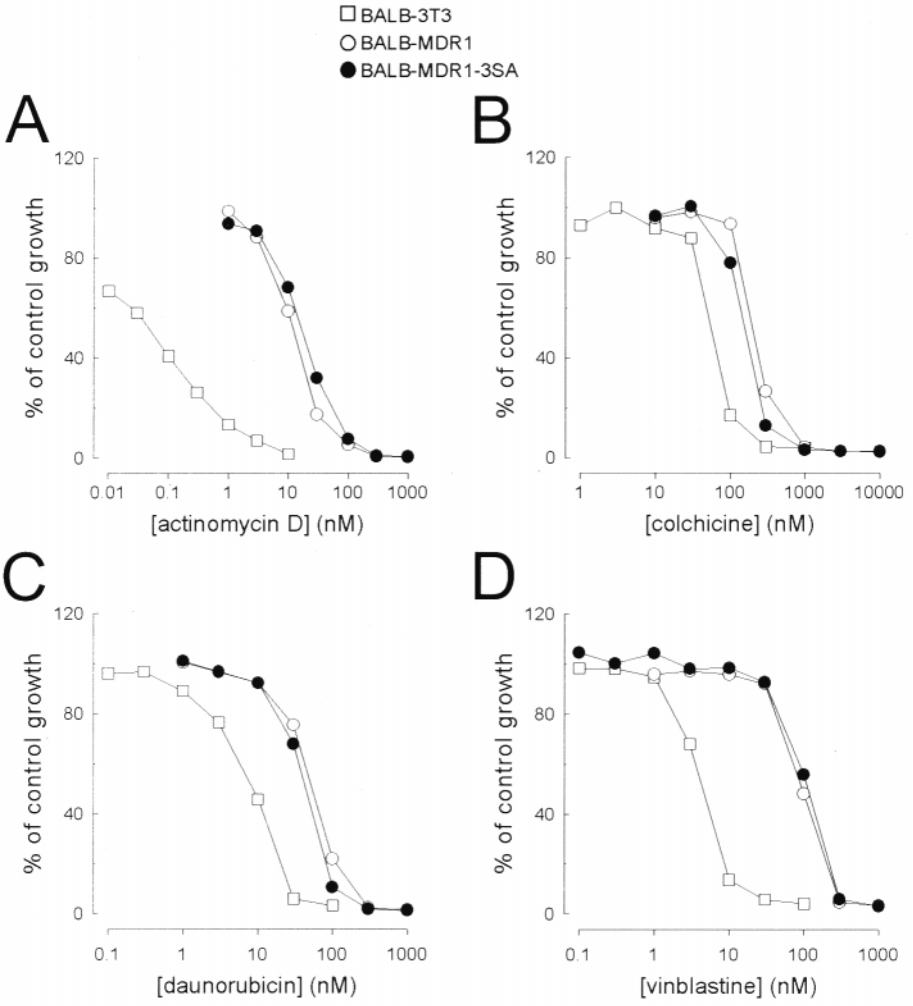

FIG. 4. Sensitivity of BALB-3T3, BALB-MDR1, and BALB-MDR1–3SA cells to cytotoxic agents. Growth inhibition was determined following continuous exposure to drugs, and expressed as a percentage of the growth in control, untreated, cells (average of 0.97×10^6 cells at the end of the experiment). Experiments with similar results were performed three times.

3SA was undistinguishable. However, we found that R123 efflux in BALB-MDR1–3SA cells was more sensitive to inhibition by vinblastine than that of BALB-MDR1 cells. The rate constant for R123 efflux by exposure to 2.5 μM vinblastine was reduced more in BALB-MDR1–3SA than in BALB-MDR1 cells (75 ± 2 vs 47 ± 3%, respectively, N = 3 each, $P < 0.05$). The inhibition by 25 μM vinblastine was essentially complete in both cell lines (>98%). These results suggest that there may be subtle changes in the transport properties of the PKC-defective phosphorylation mutant that require further study.

Effects of PKC Inhibitors in Cells Expressing the PKC Phosphorylation-Defective Mutant of MDR1

Figure 5A illustrates the rapid effect of staurosporine on R123 efflux in BALB-MDR1–3SA cells. The time course of the effect of staurosporine was very similar to that in BC19/3 cells (see Fig. 2), i.e. staurosporine rapidly reduced R123 efflux in a reversible manner. Figure 5B shows that the rapid decrease in the rate constant for R123 efflux produced by staurosporine was similar in BALB-MDR1 and BALB-MDR1–3SA cells. The rapid decrease in the rate constant for R123 efflux by chelerythrine (100 μM) was also similar in BALB-MDR1 and BALB-MDR1–3SA cells (39 ± 14 and 29 ± 9%, respectively). These results indicate that the rapid effect of the blockers is independent of MDR1 phosphorylation.

To confirm the results, we determined the effect of staurosporine on the transport of calcein-AM, a known MDR1 substrate [22, 23, 27]. It has been demonstrated that the rate of calcein accumulation is inversely related to P-glycoprotein transport [22, 23]. Calcein-AM is essentially non-fluorescent, but upon de-esterification by cell esterases, the highly-fluorescent calcein is produced (the free acid calcein is not an MDR1 substrate). In the presence of functional MDR1, the rate of increase in calcein fluorescence is reduced because of an increase in P-glycoprotein-associated calcein-AM efflux (a significant fraction of calcein-AM is extruded before de-esterification). A typical experiment is shown in Fig. 6. The rate of increase in calcein fluorescence was decreased by MDR1 expression, and the addition of the nonfluorescent MDR1 modulator verapamil increased the rate of change in fluorescence only in MDR1 expressing cells. The rate of increase in fluorescence was similar in BALB-3T3 and BALB-MDR1 cells after the addition of 100 μM verapamil. This concentration

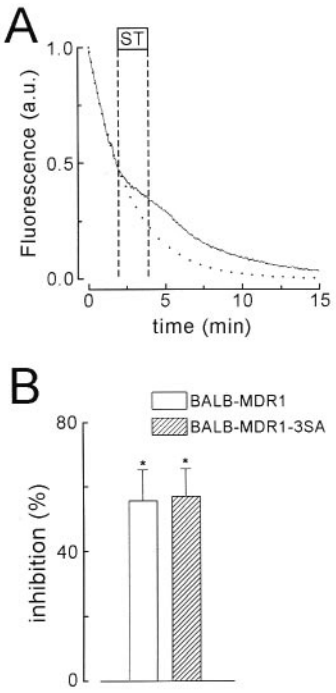

FIG. 5. Rapid inhibition of R123 efflux by staurosporine. (A) Effect of staurosporine in BALB-MDR1–3SA cells. The experiment was performed essentially as described in the legend of Fig. 2, but staurosporine (ST) was used at a concentration of 3 μM. The dotted line is the best fit of Equation 1 to the data before the addition of staurosporine. (B) Average inhibition by 3 μM staurosporine in BALB-MDR1 and BALB-MDR1–3SA cells. Averages of 5–6 experiments, similar to that in panel A, are presented (means ± SEM). Average rate constants for R123 efflux in BALB-MDR1 and BALB-MDR1–3SA cells were 0.42 ± 0.04 and 0.40 ± 0.05, respectively (means ± SEM). Key: (*) indicates statistical significance ($P < 0.05$) compared with the corresponding control, in the absence of PKC inhibitors.

of verapamil was the maximal inhibitory concentration (data not shown). The verapamil-sensitive rate of increase in fluorescence upon the addition of 2 μM calcein-AM was similar in BALB-MDR1 and BALB-MDR1–3SA cells (54 ± 3 vs 60 ± 2%, respectively, N = 6 and 7, respectively). Figure 6B shows that staurosporine produced a rapid increase in the rate of elevation in fluorescence in BALB-MDR1–3SA cells (similar results were obtained in BALB-MDR1 cells; data not shown). To rule out that changes in calcein fluorescence are due to effects on esterase activity, as opposed to calcein-AM transport, we studied the effects of verapamil on the increase in cell fluorescence upon exposure to BCECF-AM. BCECF-AM is not an MDR1 substrate [14], and elevation in BCECF and calcein fluorescence in cells is dependent on the esterase activity [23]. The rate of increase in BCECF fluorescence in BALB-3T3, BALB-MDR1, and BALB-MDR1–3SA upon exposure to 0.5 μM BCECF-AM was undistinguishable, and was not affected by 100 μM verapamil. Together with the R123 efflux data, the calcein-AM results indicate that the rapid effect of PKC blockers on drug transport is independent of MDR1 phosphorylation. In support of this conclusion, staurosporine reduced photoaffinity labeling of MDR1 and MDR1–3SA by [^{3}H]azidopine (Fig. 7A). These observations indicate that staurosporine (also chelerythrine, see Fig. 7B below) interacts with both MDR1 and MDR1–3SA. In summary, our results indicate that at least part of the decrease in drug transport by PKC blockers is independent of MDR1 phosphorylation. It seems likely that direct competition with transported drugs for binding to MDR1 is responsible for the inhibitory effect.

Evidence for an Effect of Safingol without Decreasing Photolabeling of MDR1 by [^{3}H]Azidopine

From the results above, we cannot rule out that PKC inhibitors also block MDR1-associated drug transport by a mechanism additional to direct competition for binding to MDR1. In this context, it has been shown that some PKC blockers, such as safingol and a myristoylated PKC inhibitory peptide, that do not compete out MDR1 photolabeling with [^{3}H]azidopine, do decrease MDR1-associated drug transport and MDR1 phosphorylation [28, 29]. Therefore, it is possible that inhibition of PKC (i) blocks MDR1-associated drug transport by reducing MDR1 phosphorylation, and/or (ii) decreases drug transport by decreasing PKC activity and modifying a signaling pathway that regulates MDR1 independently of its phosphorylation (e.g. reducing the phosphorylation of a regulatory protein). To differentiate between these possibilities, we determined the effects of safingol on calcein transport in BALB-MDR1 and

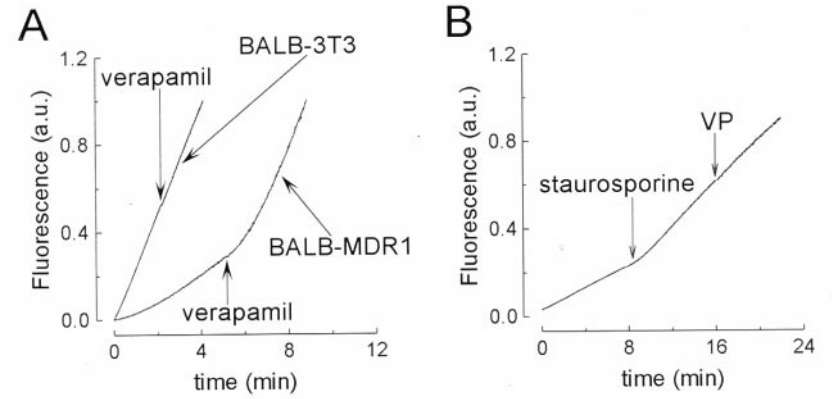

FIG. 6. Rapid increase in calcein accumulation by staurosporine. (A) Effect of MDR1 expression on the rate of increase of calcein fluorescence. Calcein-AM (1 μM) was added at $t = 0$ to cells in suspension. Verapamil (100 μM) was added when indicated. The traces are representative of at least four similar experiments for each cell line. (B) Effect of staurosporine on calcein accumulation in BALB-MDR1–3SA cells. The experimental protocol was similar to that in A. Staurosporine (3 μM) and verapamil (100 μM) were added when indicated. The trace is representative of three similar experiments.

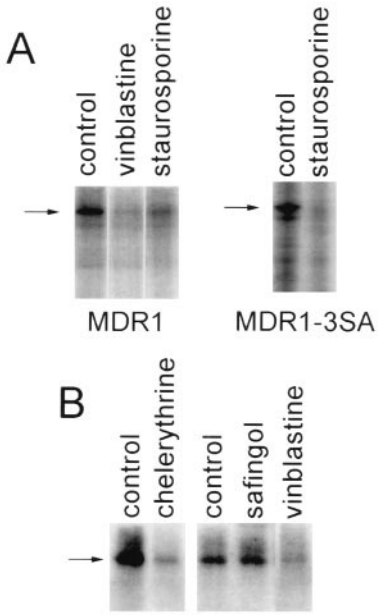

FIG. 7. Effects of PKC inhibitors on photoaffinity labeling of P-glycoprotein by [^{3}H]azidopine. (A) Effect of staurosporine (30 μM) on *"in vivo"* MDR1 and MDR1–3SA photolabeling by [^{3}H]azidopine. The gels are representative of 2–3 similar experiments. Experiments were carried out on BALB-MDR1 and BALB-MDR1–3SA cells. (B) Effects of chelerythrine (100 μM) and safingol (50 μM) on *"in vitro"* MDR1 photolabeling by [^{3}H]azidopine of BALB-1000 membranes. Vinblastine (100 μM) was used in panels A and B as a positive control to compete MDR1 photolabeling by [^{3}H]azidopine.

BALB-MDR1–3SA cells. We confirmed the observation that safingol, an analogue of sphingosine, does not compete MDR1 photolabeling by [^{3}H]azidopine [29] (Fig. 7B). These *in vitro* photoaffinity labeling experiments were performed on membranes of BALB-1000 cells because the photolabeling signal obtained using BALB-MDR1 and BALB-MDR1–3SA was very weak due to the low MDR1 expression. Figure 8A shows a representative experiment illustrating the lack of a rapid effect of safingol on calcein accumulation in BALB-MDR1–3SA cells. Also, safingol had no effects on BALB-MDR1 cells in similar experiments (data not shown). On the other hand, exposure to safingol for 2 hr increased the rate of calcein accumulation. A typical experiment on BALB-MDR1–3SA cells is shown in Fig. 8B, and average data in BALB-MDR1 and BALB-MDR1–3SA cells are shown in Fig. 8C. Safingol had no effects on the rate of increase in BCECF fluorescence (data not shown). The results indicate that MDR1 inhibition by long-term exposure to safingol is independent of MDR1 phosphorylation because it was also observed in BALB-MDR1–3SA cells.

DISCUSSION

Although it is clear that PKC inhibitors reduce P-glycoprotein phosphorylation and P-glycoprotein-associated drug transport [4, 6–9], the mechanisms of the effect on drug

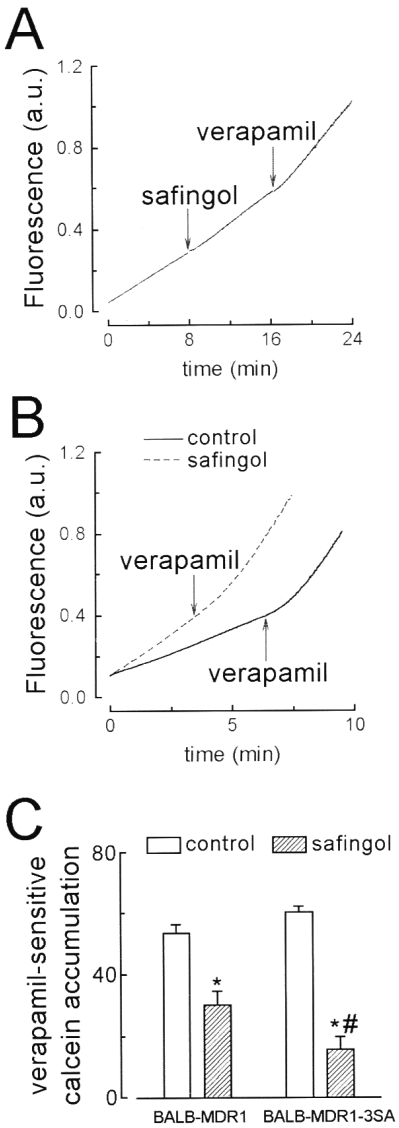

FIG. 8. Effects of safingol on calcein accumulation. (A) Absence of rapid effects of safingol on calcein accumulation in BALB-MDR1–3SA cells. The experimental protocol was similar to that in Fig. 6. (B) Increase of calcein accumulation by a 2-hr exposure to safingol in BALB-MDR1–3SA cells. Calcein-AM, 0.5 μM, was added to both the control and the safingol-treated BALB-MDR1–3SA cells. (C) Effects of a 2-hr exposure to safingol on calcein accumulation in BALB-MDR1 and BALB-MDR1–3SA cells. Data (means ± SEM, N = 6–8) are expressed as a percentage of the ratio: verapamil-sensitive calcein accumulation rate/rate of calcein accumulation in the presence of verapamil. The values for BALB-3T3 cells were not different from 100%, and are not shown (see Fig. 6). Calcein-AM was added to a final concentration of 2 μM. The average rate of increase in calcein fluorescence in the presence of verapamil was similar in BALB-MDR1 and BALB-MDR1–3SA cells [482 and 429 F (a.u.)/min/10^6 cells/mL, respectively]. Key: (*) indicates statistical significance ($P < 0.05$) compared with the corresponding control, in the absence of safingol; and (#) indicates statistical significance ($P < 0.05$) compared with BALB-MDR1 cells treated with safingol. The concentrations of safingol and verapamil were 50 and 100 μM, respectively. Fluorescence was measured as described in the legend of Fig. 6.

transport are not clear [4, 11–13]. The use of PKC blockers that compete the specific [^{3}H]azidopine photolabeling of P-glycoprotein and/or specific vincristine and vinblastine binding to P-glycoprotein has made the results of the studies difficult to interpret [4, 11–13]. It is not clear whether the decrease in drug transport is due to the decrease in P-glycoprotein phosphorylation caused by inhibition of PKC, or to direct competition for binding to P-glycoprotein between the transported drugs and PKC blockers.

Studies on drug accumulation and modulation of P-glycoprotein phosphorylation by PKC blockers and stimulators have shown a good correlation between multidrug resistance and the state of P-glycoprotein phosphorylation [4, 6–10]. In agreement with these observations, we found that the PKC blockers staurosporine, calphostin C, and chelerythrine increased drug accumulation only in cells expressing MDR1. We also showed that the PKC blockers acted by decreasing drug efflux. Although the simplest explanation is that reducing MDR1 phosphorylation decreases MDR1-associated drug transport, several findings are inconsistent with this view. We found that several PKC blockers produced an inhibition of R123 and calcein-AM transport that seemed too fast (<1 min) to be due to dephosphorylation of MDR1. The rate of dephosphorylation/phosphorylation of P-glycoprotein is approximately 30 min [25]. The reversal of the rapid inhibition of drug transport was also very rapid (started at *ca.* 2 min) when compared with the P-glycoprotein phosphorylation turnover. In fact, the effects of the PKC blockers on MDR1-associated R123 efflux were similar to those of nonfluorescent drugs (e.g. verapamil and vinblastine) that compete with R123 [20]. The conclusion that the rapid block of MDR1-associated drug transport by PKC inhibitors is due to competition for binding is supported by the observation that PKC blockers inhibit photolabeling of P-glycoprotein by [^{3}H]azidopine as well as drug binding to P-glycoprotein [11–13; present work]. More importantly, the rapid effects of PKC inhibitors on drug transport were observed in cells expressing a PKC phosphorylation-defective MDR1 mutant in which Ser^{661}, Ser^{667}, and Ser^{671} of the linker region of MDR1 were replaced with non-phosphorylatable alanine residues. Ser^{661}, Ser^{667}, and Ser^{671} have been identified as the residues phosphorylated by PKC [3, 4, 26; confirmed in this work]. In addition, safingol, a PKC inhibitor that does not interact directly with MDR1, has no rapid effect on MDR1-associated drug transport. Based on the observations above, we conclude unambiguously that many PKC inhibitors reduce MDR1-associated drug transport by a mechanism independent of MDR1 phosphorylation. Direct competition with transported drugs for binding to MDR1 is the most likely mechanism. It also has been shown that analogs of staurosporine with low potency at inhibiting PKC increase drug accumulation in MDR1 expressing cells [13].

The cell lines that we obtained without selection with chemotherapeutic agents expressed MDR1 or MDR1–3SA at low levels, and as a consequence we found a linear relationship between P-glycoprotein expression and drug transport (Fig. 3). At high levels of P-glycoprotein expression, drug transport changes as a function of P-glycoprotein expression are small, and the sensitivity of the transport assays may be reduced [20]. Thus, our cell lines are ideal for transport studies. A drawback of the use of cells with low P-glycoprotein expression is that estimations of the phosphorylation of MDR1 and MDR1–3SA are difficult, preventing determinations of phosphorylation/dephosphorylation turnover and of effects of blockers on phosphorylation.

Although the concentrations of the PKC inhibitors used in the present study may seem rather high, they are within the range used in previous publications [7, 10, 13, 30, 31]. In addition, we have observed partial effects at concentrations lower than those shown in Results (e.g. 0.3 μM staurosporine, 1–10 μM chelerythrine, and 1 μM calphostin C). The variability in the effective inhibitor concentrations in different cell types could be explained by differences in permeability to the blockers, partition in the plasma membrane, and/or expression of PKC isoforms.

In addition to the elucidation of the mechanism of the rapid effects of PKC inhibitors, we tried to determine whether PKC blockers have effects on MDR1-associated drug transport that are independent of direct competition with transported drugs for binding. To answer this question, we confirmed previous observations that safingol does not compete with photolabeling of MDR1 by [^{3}H]azidopine, and showed that a 2-hr exposure to the PKC blocker did reduce drug transport, as previously described [29]. As mentioned above, safingol had no rapid effect on calcein-AM transport. The inhibition by safingol in cells expressing the PKC phosphorylation-defective MDR1 mutant indicates that most of the safingol effect is due to inhibition of MDR1-associated drug transport independent of direct MDR1 phosphorylation effects.

It has been shown that manipulation of PKC-α expression affects MDR1-mediated drug transport [32, 33], but recent studies [34, 35] and the present work strongly suggest that phosphorylation of MDR1 by PKC does not play a major role in the regulation of drug transport by MDR1. The inhibition of MDR1-mediated drug transport by safingol in BALB-MDR1–3SA cells suggests that alterations in PKC-α expression modulate MDR1-mediated drug transport by changing the phosphorylation of one or more proteins. Our observations are also consistent with both the lack of significant effects on drug transport of the substitution of potentially phosphorylatable residues [34, 35], and the reversal of drug resistance by PKC inhibitors that do not interact with P-glycoprotein [28, 29, 36, 37]. The speculation that the PKC pathway modulates MDR1-mediated drug transport indirectly is based on the results using safingol (see Table 1 for a summary) as well as results by others using a myristoylated PKC-α peptide inhibitor [28] and manipulation of PKC-α expression/activity [32, 33]. Therefore, the available information supports a role of the PKC pathway in the control of substrate transport by MDR1. However, the possible contribution of other mech-

TABLE 1. Summary of results in BALB-3T3, BALB-MDR1, and BALB-MDR1-3SA

	BALB-3T3	BALB-MDR1	BALB-MDR1-3SA
MDR1 expression	No	Yes (wild-type)	Yes (PKC-mutant)
Drug transport rate	Low	High, same in both cell lines	
Competition by pKCls of specific photolabeling	N/A	Yes, observed in both cell lines	
"Long-term" effect of safingol	ND	Yes, observed in both cell lines	

PKC-mutant: MDR1-3SA mutant, Ser^{661}, Ser^{667}, and Ser^{671} substituted by alanine residues. PKCls: PKC inhibitors staurosporine and chelerythrine. N/A: not applicable, no specific photolabeling when [^{3}H]azidopine is present. ND: not determined because the verapamil-sensitive rate of increase in calcein florescence under control conditions is not different from zero.

anisms (e.g. changes in membrane fluidity by safingol) cannot be ruled out.

An unexpected result was the small but significant increase in the block of calcein-AM transport by long-term exposure to safingol in BALB-MDR1–3SA as compared with BALB-MDR1 cells (see Fig. 8C). Although we do not have a definitive explanation for this observation, one possibility is that MDR1 phosphorylation by PKC has a small inhibitory effect on calcein-AM transport.

Although studying the effects of substitutions in the PKC-phosphorylatable residues was not the major aim of the present work, our results strongly support the notion that Ser [661], Ser [667], and Ser [671] are the residues phosphorylated by PKC [3, 4, 26; see Fig. 3]. Our data are also in agreement with previous publications that show little or no effect on drug transport and multidrug resistance of substitutions of the serines and threonines in the MDR1 linker region by alanine or aspartic/glutamic acid residues [27, 34, 35]. On the other hand, recent results indicate that phosphorylation of MDR1 by PKA and PKC is involved in the modulation of swelling-activated Cl^- channels [38, 39]. Although our data support the notion that phosphorylation of MDR1 by PKC does not play a major role in the regulation of drug transport, a minor role is possible. This is suggested by the effects of mutations of PKC-phosphorylatable serines on drug-stimulated MDR1 ATPase activity [27] and the increased potency of vinblastine for blocking R123 efflux in BALB-MDR1–3SA cells (see Results).

In summary, we conclude that PKC inhibitors reduce MDR-mediated drug transport by (i) direct competition with transported drugs for binding to MDR1, and (ii) indirect inhibition through a pathway that involves PKC inhibition, but is independent of MDR1 phosphorylation. We speculate that this pathway involves PKC-mediated phosphorylation of one or more proteins that modulate MDR1. The effects of the blockers on MDR1 phosphorylation do not seem to play an important role, but the PKC-signaling pathway regulates drug transport by MDR1.

We thank Drs. S. Weinman and J-T. Zhang for comments on a preliminary version of the manuscript, and Dr. P. Melera for providing us with the mammalian expression vector pLK-444. We also wish to thank J. Ehardt, J. Lock, and Dr. C. Wang for technical assistance, and M. Griffin for the flow cytometry studies performed at the Flow Cytometry and Cell Sorting Facility of the University of Texas Medical Branch. We appreciate the work of Dr. T. Pourcher in the engineering of the polyhistidine tag at the C-terminal end of the MDR1 cDNA constructs. This work was supported, in part, by American Heart Association, Texas Affiliate, Inc., Grant 96G-1613, National Cancer Institute Grant CA72783, and National Institutes of Diabetes and Kidney Diseases Grant DK-08865.

References

1. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
2. Sharom FJ, The P-glycoprotein efflux pump: How does it transport drugs? *J Membr Biol* **160:** 161–175, 1997.
3. Chambers TC, Pohl J, Raynor RL and Kuo JF, Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol Chem* **268:** 4592–4595, 1993.
4. Germann UA, Chambers TC, Ambudkar SV, Pastan I and Gottesman MM, Effects of phosphorylation of P-glycoprotein on multidrug resistance. *J Bioenerg Biomembr* **27:** 53–61, 1995.
5. Orr GA, Han EKH, Browne PC, Nieves E, O'Connor BM, Yang CPH and Horwitz SB, Identification of the major phosphorylation domain of murine mdr1b P-glycoprotein. Analysis of the protein kinase A and protein kinase C phosphorylation sites. *J Biol Chem* **268:** 25054–25062, 1993.
6. Bates SE, Lee JS, Dickstein B, Spolyar M and Fojo A, Differential modulation of P-glycoprotein transport by protein kinase inhibition. *Biochemistry* **32:** 9156–9164, 1993.
7. Chambers TC, Zheng B and Kuo JF, 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. *Mol Pharmacol* **41:** 1008–1015, 1992.
8. Meyers MB, Protein phosphorylation in multidrug resistant Chinese hamster cells. *Cancer Commun* **1:** 233–241, 1989.
9. Sato W, Yusa K, Naito M and Tsuruo T, Staurosporine, a potent inhibitor of C-kinase, enhances drug accumulation in multidrug-resistant cells. *Biochem Biophys Res Commun* **173:** 1252–1257, 1990.
10. Bates SE, Currier SJ, Alvarez M and Fojo AT, Modulation of P-glycoprotein phosphorylation and drug transport by sodium butyrate. *Biochemistry* **31:** 6366–6372, 1992.
11. Budworth J, Davies R, Malkhandi J, Gant TW, Ferry DR and Gescher A, Comparison of staurosporine and four analogues: Their effects on growth, rhodamine 123 retention and binding to P-glycoprotein in multidrug-resistant MCF-7/Adr cells. *Br J Cancer* **73:** 1063–1068, 1996.
12. Smith CD and Zilfou JT, Circumvention of P-glycoprotein-mediated multiple drug resistance by phosphorylation modulators is independent of protein kinases. *J Biol Chem* **270:** 28145–28152, 1995.

13. Wakusawa S, Inoko K, Miyamoto K-I, Kajita S, Hasegawa T, Harimaya K and Koyama M, Staurosporine derivatives reverse multidrug resistance without correlation with their protein kinase inhibitory activities. *J Antibiot (Tokyo)* **46:** 353–355, 1993.
14. Altenberg GA, Young G, Horton JK, Glass D, Belli JA and Reuss L, Changes in intra- or extracellular pH do not mediate P-glycoprotein-dependent multidrug resistance. *Proc Natl Acad Sci USA* **90:** 9735–9738, 1993.
15. Castro AF and Altenberg GA, Inhibition of drug transport by genistein in multidrug-resistant cells expressing P-glycoprotein. *Biochem Pharmacol* **53:** 89–93, 1997.
16. Lambeth JD, Burnham DN and Tyagi SR, Sphinganine effects on chemoattractant-induced diacylglycerol generation, calcium fluxes, superoxide production, and on cell viability in the human neutrophil. Delivery of sphinganine with bovine serum albumin minimizes cytotoxicity without affecting inhibition of the respiratory burst. *J Biol Chem* **263:** 3818–3822, 1988.
17. Devine SE and Melera PW, Diversity of multidrug resistance in mammalian cells. *J Biol Chem* **269:** 6133–6139, 1994.
18. Vanoye CG, Altenberg GA and Reuss L, P-glycoprotein is not a swelling-activated Cl^- channel; Possible role as a Cl^- channel regulator. *J Physiol (Lond)* **502:** 249–258, 1997.
19. Han ES, Vanoye CG, Altenberg GA and Reuss L, P-glycoprotein-associated chloride currents revealed by specific block by an anti-P-glycoprotein antibody. *Am J Physiol* **270:** C1370–C1378, 1996.
20. Altenberg GA, Vanoye CG, Horton JK and Reuss L, Unidirectional fluxes of rhodamine 123 in multidrug-resistant cells: Evidence against direct drug extrusion from the plasma membrane. *Proc Natl Acad Sci USA* **91:** 4654–4657, 1994.
21. Horton JK, Thimmaiah KN, Altenberg GA, Castro AF, Germain GS, Gowda GK and Houghton PJ, Characterization of a novel bis-acridone and comparison with PSC-833 as a potent and poorly reversible modulator of P-glycoprotein. *Mol Pharmacol* **52:** 948–957, 1997.
22. Homolya L, Hollo Z, Muller M, Mechetner EB and Sarkadi BA, New method for quantitative assessment of P-glycoprotein-related multidrug resistance in tumour cells. *Br J Cancer* **73:** 849–855, 1996.
23. Essodaigui M, Broxterman HJ and Garnier-Suillerot A, Kinetic analysis of calcein and calcein-acetoxymethylester efflux mediated by the multidrug resistance protein and P-glycoprotein. *Biochemistry* **37:** 2243–2250, 1998.
24. Butler WB, Preparing nuclei from cells in monolayer cultures suitable for counting and for following synchronized cells through the cell cycle. *Anal Biochem* **141:** 70–73, 1984.
25. Ma L, Marquardt D, Takemoto L and Center MS, Analysis of P-glycoprotein phosphorylation in HL60 cells isolated for resistance to vincristine. *J Biol Chem* **266:** 5593–5599, 1991.
26. Chambers TC, Pohl J, Glass DB and Kuo JF, Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. *Biochem J* **299:** 309–315, 1994.
27. Szabo K, Bakos E, Welker E, Muller M, Goodfellow HR, Higgins CF, Varadi A and Sarkadi B, Phosphorylation site mutations in the human multidrug transporter modulate its drug-stimulated ATPase activity. *J Biol Chem* **272:** 23165–23171, 1997.
28. Bergman PJ, Gravitt KR and O'Brian CA, An *N*-myristoylated protein kinase C-α pseudosubstrate peptide that functions as a multidrug resistance reversal agent in human breast cancer cells is not a P-glycoprotein substrate. *Cancer Chemother Pharmacol* **40:** 453–456, 1997.
29. Sachs CW, Safa AR, Harrison SD and Fine RL, Partial inhibition of multidrug resistance by safingol is independent of modulation of P-glycoprotein substrate activities and correlated with inhibition of protein kinase C. *J Biol Chem* **270:** 26639–26648, 1995.
30. Lo CF and Breitwieser GE, Protein kinase-independent inhibition of muscarinic K^+ channels by staurosporine. *Am J Physiol* **266:** C1128–C1132, 1994.
31. Shah BH, Shamim G, Khan S and Saeed SA, Protein kinase C inhibitor, chelerythrine, potentiates the adrenaline-mediated aggregation of human platelets through calcium influx. *Biochem Mol Biol Int* **38:** 1135–1141, 1996.
32. Ahmad S and Glazer RI, Expression of the antisense cDNA for protein kinase Cα attenuates resistance in doxorubicin-resistant MCF-7 breast carcinoma cells. *Mol Pharmacol* **43:** 858–862, 1993.
33. Yu G, Ahmad S, Aquino A, Fairchild CR, Trepel JB, Ohno S, Suzuki K, Tsuruo T, Cowan KH and Glazer RI, Transfection with protein kinase C α confers increased multidrug resistance to MCF-7 cells expressing P-glycoprotein. *Cancer Commun* **3:** 181–189, 1991.
34. Germann UA, Chambers TC, Ambudkar SV, Licht T, Cardarelli CO, Pastan I and Gottesman MM, Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. *J Biol Chem* **271:** 1708–1716, 1996.
35. Goodfellow HR, Sardini A, Ruetz S, Callaghan R, Gros P, McNaughton PA and Higgins CF, Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. *J Biol Chem* **271:** 13668–13674, 1996.
36. Sachs CW, Ballas LM, Mascarella SW, Safa AR, Lewin AH, Loomis C, Carroll FI, Bell RM and Fine RL, Effects of sphingosine stereoisomers on P-glycoprotein phosphorylation and vinblastine accumulation in multidrug-resistant MCF-7 cells. *Biochem Pharmacol* **52:** 603–612, 1996.
37. Bergman PJ, Gravitt KR, Ward NE, Beltran P, Gupta KP and O'Brian CA, Potent induction of human colon cancer cell uptake of chemotherapeutic drugs by *N*-myristoylated protein kinase C-α (PKC-α) pseudosubstrate peptides through a P-glycoprotein-independent mechanism. *Invest New Drugs* **15:** 311–318, 1997.
38. Bond TD, Valverde MA and Higgins CF, Protein kinase C phosphorylation disengages human and mouse-1a P-glycoproteins from influencing the rate of activation of swelling-activated chloride currents. *J Physiol (Lond)* **508:** 333–340, 1998.
39. Vanoye CG, Castro AF, Pourcher T, Reuss L and Altenberg GA, Phosphorylation of P-glycoprotein by PKA and PKC modulates swelling-activated Cl^- currents. *Am J Physiol* **276:** C370–C378, 1999.