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P-Glycoprotein-independent Decrease in Drug Accumulation by Phorbol Ester Treatment of Tumor Cells

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ABSTRACT. The effect of a change in the phosphorylation state of the drug transporter P-glycoprotein (P-gp) on its drug transport activity was studied for the substrates daunorubicin (DNR), etoposide (VP-16), and calcein acetoxymethyl ester (Cal-AM). Phorbol ester (PMA), added to stimulate phosphorylation of P-gp by protein kinase C (PKC), caused a decrease in the cellular accumulation of DNR and VP-16, both in multidrug-resistant (MDR) P-gp-overexpressing cells and in wild-type cells. Since treatment of cells with kinase inhibitor staurosporine (ST) reversed this effect of PMA and the non-PKC-stimulating phorbol ester 4α-phorbol, 12,13-didecanoate (4αPDD) did not result in a decreased DNR accumulation, we conclude that this effect is the result of kinase activity. The concentration dependence of the inhibition of P-gp by verapamil (Vp) was not influenced by PMA. Accumulation of the P-gp substrate Cal-AM was not influenced by PMA in wild-type cells. Therefore, Cal-AM was used to study the effect of PMA-induced phosphorylation of P-gp on its transport activity. Activation of PKC with PMA or inhibition of protein phosphatase 1/2A (PP1/PP2A) with okadaic acid (OA) did not affect the accumulation of Cal-AM in the MDR cells or wild-type cells. The kinase inhibitor ST increased the Cal-AM accumulation only in the MDR cells. Neither stimulating PKC with PMA nor inhibiting PP1/PP2A with OA led to a decreased inhibition of P-gp by ST, indicating that ST inhibits P-gp directly. From these experiments, we conclude that PKC and PP1/PP2A activity do not regulate the drug transport activity of P-gp. However, these studies provide evidence that PMA-induced PKC activity decreases cellular drug accumulation in a P-gp-independent manner. BIOCHEM PHARMACOL 54;7:791–799, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. multidrug resistance; P-glycoprotein; drug transport; protein phosphorylation; phorbol ester

The occurrence of drug-resistant cells is a major obstacle in the treatment of cancer with chemotherapy. Often, the patient's tumor becomes resistant not only to the drug used in the therapy but to a broad spectrum of unrelated cytotoxic drugs as well. In the laboratory, exposure of drug-sensitive cells to increasing concentrations of anticancer drugs such as vinblastine or doxorubicin may result in cancer cells with a multidrug-resistant (MDR†) phenotype. When human tumor cells are selected in this way, they often overexpress the MDR1 gene, which codes for P-glycoprotein (P-gp), a membrane-bound ATP-dependent multidrug export protein [1].

Several research teams have shown that P-gp can be phosphorylated [2]. Serine and threonine phosphorylating kinase (PKC) [3] has been identified to phosphorylate P-gp [4–8]. *In vitro* cAMP-dependent kinase (PKA) also phosphorylated P-gp [8]. Using the potent phosphatase inhibitor okadaic acid (OA) [9], it was shown that protein phosphatase 1/2A (PP1/PP2A) was involved in the dephosphorylation of P-gp [5, 7, 10].

It has been suggested that drug transport activity could be modulated by changing the state of phosphorylation. Stimulation of PKC with phorbol ester (PMA) in KB-V1 cells led to an increased phosphorylation of P-gp and a decreased accumulation of vinblastine. Inhibition of PKC with protein kinase inhibitors led to a decreased phosphorylation of P-gp and an increased accumulation of vinblastine [5, 11]. Transfection of P-gp-expressing cells with the gene coding for PKCα-increased drug resistance increased the phosphorylation of P-gp and decreased vinblastine accumulation [12]. These studies suggest that increased phosphorylation of P-gp stimulates drug transport activity, at least for vinblastine.

However, the correlation between P-gp phosphorylation and active drug transport is not complete. Using several

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† *Abbreviations:* BCECF-AM, 2′7′-bis-(2-carboxyethyl)-5′6′-carboxyfluorescein acetoxymethyl ester; Cal-AM, calcein acetoxymethyl ester; DMEM, Dulbecco's minimal essential medium; DNR, daunorubicin; FCS, fetal calf serum; MDR, multidrug resistance; NaF, sodium fluoride; OA, okadaic acid; 4αPDD, 4α-phorobol, 12,13-didecanoate; P-gp, P-glycoprotein; PKA, protein kinase A; PKC, protein kinase C; PMA, 12-O-tetradecanoyl phorbol 13-acetate; PP1/PP2A, protein phosphatase 1/2A; ST, staurosporine; Vp, verapamil; VP-16, etoposide.

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staurosporine derivatives, Miyamoto *et al.* [13, 14] were not able to find a correlation between the degree of PKC inhibition and P-gp inhibition. Verapamil (Vp), an inhibitor of P-gp-mediated drug transport, also stimulated the phosphorylation of P-gp [15]. Waldler and Yang [16] reported that various derivatives of forskolin, which stimulated PKA and thereby P-gp phosphorylation, did not stimulate P-gp pumping. Instead, an inhibition of P-gp was found with the lipophilic forskolin derivatives. Recently, Germann *et al.* [17] found no difference in resistance between a cell line transfected with wild-type P-gp and one transfected with a phosphorylation-defective form of the human P-gp.

The aim of the present study was to examine whether protein kinases or protein phosphatases regulate transport activity of P-gp. MDR KB-V1 cells were used, as their phosphorylation of P-gp is well documented [4–8, 10, 18, 19]. In addition, the stable MDR1-transfected cell line S1(1.1) was used. To modulate the phosphorylation of P-gp, we focused on the compounds PMA, OA, and staurosporine (ST), since for these compounds modulation of the state of phosphorylation of P-gp was reported [5–7, 10, 20]. The effect of modulating the kinase or phosphatase activity on the cellular accumulation of the P-gp substrates daunorubicin (DNR), etoposide (VP-16), and calcein acetoxymethyl ester (Cal-AM) was investigated in wild-type and MDR cells.

MATERIALS AND METHODS

Chemicals

Vp, ST, PMA, 4α-phorbol, 12,13-didecanoate (4αPDD), sodium fluoride (NaF), dibutyryl cAMP, diltiazem, colchicine, vincristine sulfate, bovine albumin fraction IV (BSA), and nigericin were obtained from Sigma (St. Louis, MO, USA), OA was from Boehringer Mannheim (Almere, the Netherlands), and PSC833 was obtained from Sandoz (Basle, Switzerland). Vinblastine was obtained from Eli Lilly (Nieuwegein, the Netherlands). [^{3}H]DNR hydrochloride (59.2 GBq/mmol) was obtained from Dupont de Nemours ('s-Hertogenbosch, the Netherlands), and [^{3}H]VP-16 (33.3 GBq/mmol) was from Moravek Biochemicals Inc. (Brea, CA, USA). Cal-AM, calcein, and 2′7′-bis-(2-carboxyethyl)-5′6′-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were obtained from Molecular Probes (Eugene, OR, USA). Cell culture media and supplements were obtained from Flow (Irvine, UK), and culture plastics were from Nunc (Roskilde, Denmark). Fetal calf serum (FCS) was from Gibco (Paisley, UK), and HEPES was from Serva (Heidelberg, Germany). Glass fiber filter discs (2.5 cm in diameter) were from Whatman (Kent, UK).

Cell Culture

Wild-type KB-3-1 cells and the MDR variants KB-8-5 and KB-V1 have been described by Shen *et al.* [21]. KB-8-5 cells were cultured in the presence of 0.01 μg/mL colchicine, and KB-V1 cells were maintained in the presence of 0.2 μM vinblastine. S1(1.1) cells, which are SW1573 cells stably transfected with the MDR1 gene, were supplied by Dr. F. Baas (The Netherlands Cancer Institute, Amsterdam, the Netherlands) and cultured in the presence of 0.05 μM vincristine. Wild-type cells SW1573 have been described previously [22]. Cells were cultured in a humidified atmosphere of 5% CO_2 in Dulbecco's minimal essential medium (DMEM) with 20 mM HEPES (pH 7.4) and supplemented with 7.5% heat-inactivated FCS. One to three days before the experiments, the MDR cells were placed in medium without drug. Subsequently, nearly confluent monolayers of cells were harvested (25 mg/L trypsin, 4 g/L EDTA, and 10 mM HEPES), washed, and kept on ice in medium A with 5% FCS. Medium A is a PBS-based medium supplemented with amino acids for MEM with 5.5 mM glucose, 4 mM L-glutamine, 20 mM HEPES 20, without bicarbonate (pH 7.4). Viability of the cells exceeded 95% as determined by trypan blue exclusion.

Flow-through Experiments

Flow-through experiments were performed as previously described by Spoelstra *et al.* [23] and Mülder *et al.* [24]. One day before the experiments, cells ($10–15 \times 10^6$) were grown in monolayer on the bottom plate of the flow-through chamber. On the day of the experiment, this bottom plate was installed in the flow-through system. The system is configured with an HPLC pump (Gilson, Villiers, France, model 302, 5.S pump head) at the inlet and a fluorescence monitor (type 821-FP, Jasco, Hachioji City, Japan) at the outlet of the chamber (chamber volume 0.5–1 mL). The system was kept at 37°, and the cells were superfused with 0.5 μM DNR (in medium A with 5% FCS) at 200 μL/min. The DNR fluorescence of the medium leaving the chamber was monitored continuously by using excitation/emission wavelengths of 480/560 nm. When a steady state was reached, i.e. where the fluxes of DNR into and out of the cells in the chamber were equal, 100 μL of 250 μM Vp dissolved in the flow medium was injected into the chamber, and its effect on the steady-state concentration of DNR in the absence and presence of 100 nM PMA was studied.

Cellular [^{3}H]DNR Accumulation

[^{3}H]DNR accumulation assays were performed by incubating 0.2×10^6 cells in 1 mL of medium A with 5% FCS and 2 μM [^{3}H]DNR. Samples were prepared on ice, and the assay was started by putting the samples in a water bath of 37°. After 90 min under constant shaking, the samples were put on ice, and the cells spun down and washed with 2 mL of ice-cold PBS with 0.2% FCS. Then the pellets were transferred to counting vials, and the radioactivity was measured. Alternatively, the cell suspensions were filtered under vacuum suction over GF/C filters and washed four times with 2 mL of ice-cold washing buffer, after which the

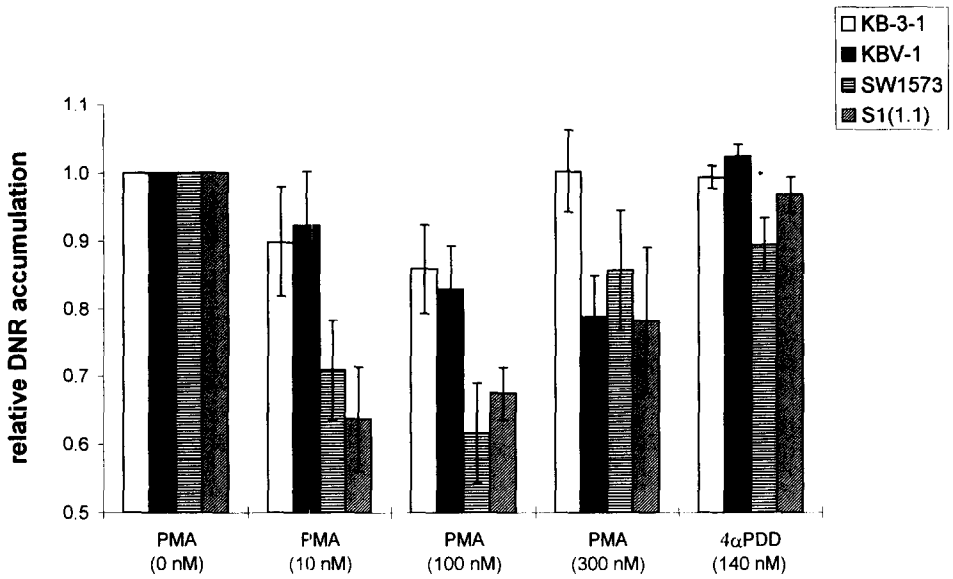

FIG. 1. The concentration-dependent effect of PMA and 4αPDD (PDD) on [^{3}H]DNR accumulation measured in wild-type cells and MDR cells. Cells were incubated in medium containing 2 μM [^{3}H]DNR and the indicated concentrations of phorbol ester for 90 min. The cells were then washed, and the cellular [^{3}H]DNR content was measured. The accumulation is expressed as [^{3}H]DNR accumulation relative to the control without phorbol ester. The absolute accumulations without PMA, given in pmol/10^6 cells, were 947 ± 119, 1021 ± 103, 342 ± 56 and 168 ± 26 for KB-3-1, SW1573, S1(1.1), and KB-V1, respectively. Data are given as the mean ± SEM of at least three independent experiments.

radioactivity on the filters was counted. The washing buffer contained 1 mg/mL BSA and 1 μM of nonradioactive DNR in PBS. To reduce binding of labeled [^{3}H]DNR, the filters were incubated in washing buffer prior to the experiment. The DNR accumulation was calculated after correction for the binding of DNR either to the walls of the incubation vial or to the filters. Differences were tested with a two-tailed paired Student's *t* test.

Cellular [^{3}H]VP-16 Accumulation

[^{3}H]VP-16 accumulation assays were performed in a similar way as for [^{3}H]DNR. Cells (0.5×10^6) in a total volume of 400 μL of medium A with 5% FCS and 0.25 μM [^{3}H]VP-16 were incubated for 60 min at 37° and shaken constantly. The cellular [^{3}H]VP-16 accumulation was calculated in a similar way as for [^{3}H]DNR.

Cellular Cal-AM Accumulation

Cal-AM accumulation assays were performed by incubating 0.5×10^6 cells/mL with 0.5 μM Cal-AM in medium A with 5% FCS. The samples were prepared on ice, and the accumulation was started by putting the samples in a water bath of 37°. After 30 min of constant shaking, the cells were put on ice, washed, and resuspended in PBS. The cellular calcein fluorescence was measured with a spectrofluorometer (FluoroMax, SPEX Industries, Edison, NJ, USA) using an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

Time-resolved experiments were started by adding 100 μL of a prewarmed (5–10 min) cell suspension of 0.2×10^6 cells to 1900 μL of medium A (5% FCS) with Cal-AM (final concentration, 0.5 μM). The intracellular Cal-AM concentration was monitored by measuring the rate of calcein production [25]. Modulators were added in such a volume (<60 μL) that the Cal-AM concentration changed less than 3%.

pH Measurement

The intracellular pH was measured by using the pH-sensitive probe BCECF in a similar way to that described by Spoelstra *et al.* [23]. In short, cells were loaded for 30 min at 37° with BCECF-AM (5 μM in PBS). After washing, the cells were incubated in medium A with 5% FCS with or without modulators for 15–25 min at 37°. Immediately after washing, the BCECF fluorescence was measured at 500/530 nm and at 440/530 nm (emission/excitation wavelengths). The intracellular pH was obtained by comparing the ratio of 500/440 with a pH standard curve made with cells loaded with BCECF in a medium containing 150 mM KCl and 5 μM nigericin. Differences were tested with a two-tailed paired Student's *t* test.

RESULTS

Cellular [^{3}H]DNR Accumulation

The influence of PMA on P-gp pump activity was investigated using [^{3}H]DNR as a probe. Figure 1 shows the effect of the PKC-stimulating phorbol ester PMA and the non-PKC-stimulating phorbol ester 4αPDD on [^{3}H]DNR accumulation in wild-type and MDR cells, expressed as the relative accumulation compared with the control. A PMA-induced decrease in [^{3}H]DNR accumulation was not only observed in the MDR cells KB-V1 and S1(1.1) but also in the wild-type cells KB-3-1 and SW1573. The effects were greatest at 100 nM PMA, while a significant ($p < 0.05$) decrease in DNR accumulation was found in all cell types. However, PMA had no significantly stronger effect ($p > 0.2$) on the relative accumulation in the MDR cells than in the wild-type cells. The non-PKC-stimulating phorbol ester

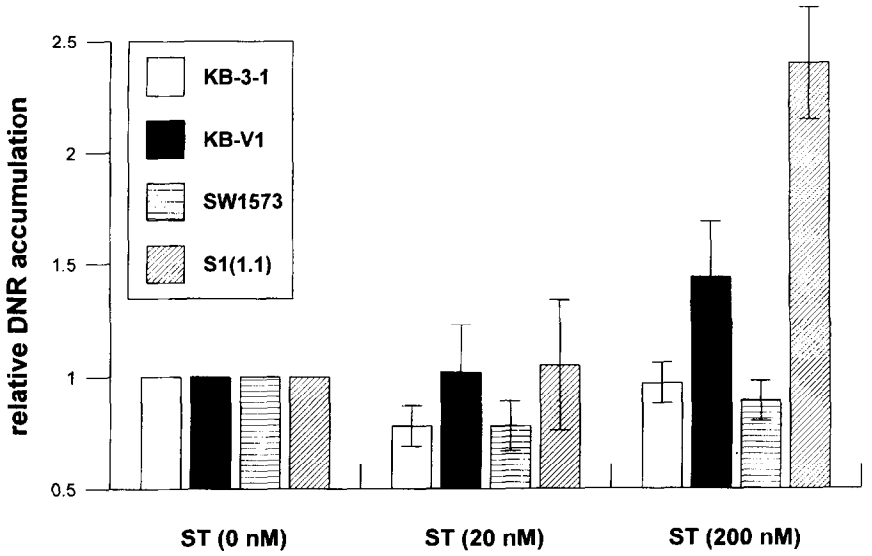

FIG. 2. The effect of 20 nM and 200 nM ST on [^{3}H]DNR accumulation measured in wild-type cells and MDR cells. Cells were incubated in medium containing 2 μM [^{3}H]DNR at the indicated concentrations of ST for 90 min. The cells were then washed, and the cellular [^{3}H]DNR content was measured. The accumulation is expressed as [^{3}H]DNR accumulation relative to the control without ST. Data are given as the mean ± SEM of at least three independent experiments.

4αPDD had no influence on the [^{3}H]DNR accumulation of the cells. In Fig. 2, the relative accumulation of DNR is given in the presence and absence of ST. At 20 nM, ST decreased the accumulation in wild-type cells, whereas at 200 nM, no difference was observed. Inhibition of P-gp was measured only at the highest concentration of ST used. Due to the effect of PMA on the wild-type cells and the observation that this effect was not always greater in the MDR cells than in the wild-type cells, these data were inconclusive as to the exact role that up-regulation or down-regulation of kinase activity may have on P-gp activity. However, we conclude that PMA has a significant effect on [^{3}H]DNR accumulation that is not related to P-gp activity.

Influence of Kinase Activity on [^{3}H]DNR Accumulation in Wild-type Cells

Since 4αPDD did not show the same effect as PMA on [^{3}H]DNR accumulation, the data in Fig. 1 appeared to show that the effect of PMA on [^{3}H]DNR accumulation in wild-type cells was probably mediated via PMA-induced PKC activity. The mechanism of PMA was further examined in wild-type cells. Figure 3 shows that the effect of PMA on [^{3}H]DNR accumulation was significantly abolished ($p < 0.05$) by the addition of ST to the medium, confirming the observations in Fig. 1 that stimulation of kinase activity was probably part of the mechanism by which PMA influenced the [^{3}H]DNR accumulation of these cells.

PMA-induced Increases in DNR Efflux Measured in a Flow-through Experiment

As an alternative method of measuring changes in cellular DNR accumulation and intracellular free drug concentration, a flow-through system was used [23, 24]. Medium containing 0.5 μM DNR was pumped over the cells. At steady state, pulses of P-gp inhibitor (100 μL with 250 μM Vp) were injected into the system. When using P-gp-

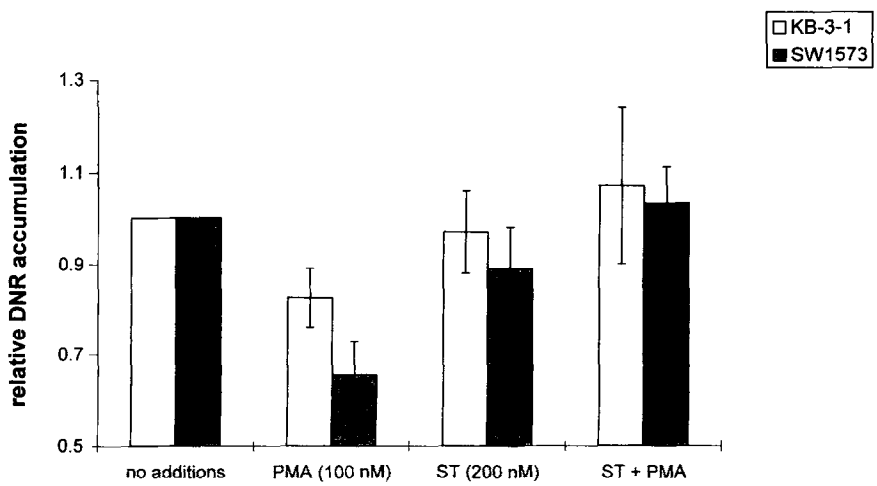

FIG. 3. The influence of ST (200 nM) on the PMA (100 nM)-induced decrease in [^{3}H]DNR accumulation, measured in wild-type cells (KB-3-1 and SW1573) is shown. Cells were incubated for 90 min in medium containing 2 μM [^{3}H]DNR at the indicated concentrations of PMA and ST. The cells were then washed, and the cellular [^{3}H]DNR content was measured. The accumulation is expressed as [^{3}H]DNR accumulation relative to the control. Data are given as the mean ± SEM of at least three independent experiments.

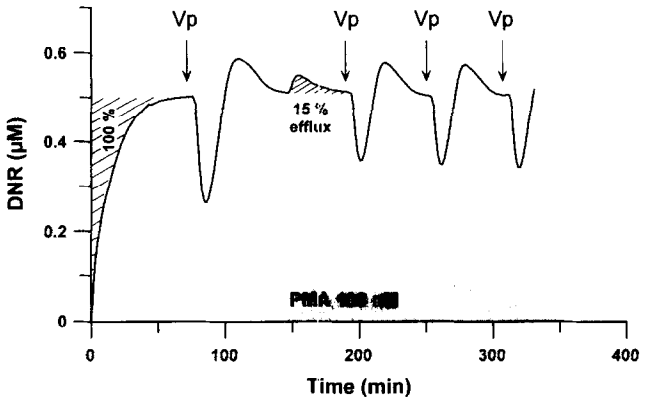

FIG. 4. Representative fluorescence trace of a flow-through experiment using 15 × 10^6 KB-V1 cells superfused with medium A + 5% FCS with 0.5 μM of DNR. 100-μL Vp injections of 250 μM were applied to induce a transient rapid and maximal inhibition of P-gp-mediated DNR pumping. Vp was injected before and during the continuous exposure of the cells to 100 nM PMA. The first shaded area indicates the initial DNR accumulation set at 100%. The second shaded area shows that 15% of the cellular DNR was effluxed from the cells upon exposure of the cells to PMA.

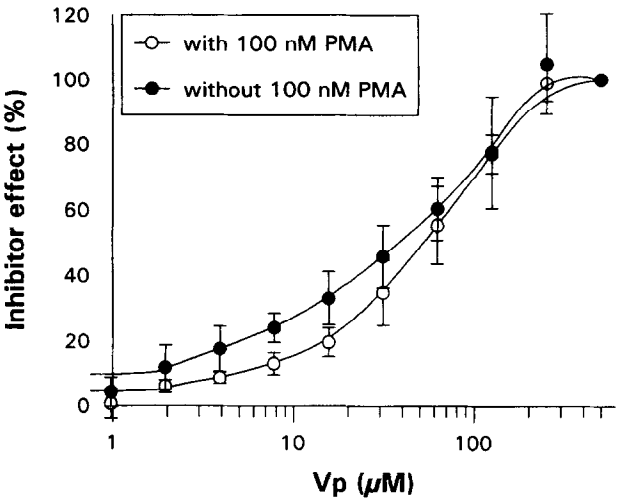

FIG. 5. The percentage of inhibition exerted by Vp on [^{3}H]DNR accumulation (90 min) in KB-V1 cells, with PMA (open circles) and without PMA (closed circles) in the medium. The accumulation was normalized by setting the [^{3}H]DNR accumulation with 500 μM Vp at 100% and the control without Vp at 0% inhibition. The absolute accumulations without PMA, given in pmol/10^6 cells, were 151 ± 37 without Vp and 1250 ± 490 with 500 μM Vp in the medium. Data are given as the mean ± SEM of three independent experiments.

expressing cells, inhibition of P-gp leads to an increased DNR uptake by the cells and consequently to a decline (dip) in DNR concentration in the effluent medium. This dip in the concentration is indicative that a DNR gradient across the plasma membrane can be maintained by P-gp activity. The depth of the dip is determined by a) the intracellular free DNR concentration before the cells were exposed to the inhibitor; b) the permeation coefficient for DNR; c) the number of cells; and d) the flow rate [24]. Cells that do not express P-gp show no dip in the DNR concentration when Vp is injected, showing the absence of P-gp activity [23]. Figure 4 shows a representative trace of an experiment performed with P-gp-overexpressing KB-V1 cells. As can be seen in this figure, injecting Vp into the system induces a decrease in the DNR concentration (the first dip).

To study the effect of PMA-induced phosphorylation of P-gp on cellular drug accumulation, 100 nM PMA was added to the flow medium as indicated in Fig. 4. The continuous presence of PMA in the medium led to an immediate efflux of DNR out of the cells, which confirmed a decrease in cellular DNR accumulation. The amount of DNR released from the cells was 15–20% of the net uptake of DNR at the beginning of the experiment, which is comparable with the decrease measured in the accumulation experiments.

To examine the effect of PMA on P-gp activity, Vp was again injected (three times) into the flow medium (see Fig. 4, the last three dips). If the PMA-induced efflux had been due to an increased pump activity, this would have resulted in a decreased intracellular DNR concentration and an increased dip depth induced by Vp. However, in the presence of PMA, the dip was less deep than before PMA was added to the medium. This decrease in the dip depth lasted for at least 3 h. Similar results were obtained with the MDR cells S1(1.1) and KB-8-5 (data not shown). The effect of PMA on these MDR cells cannot be explained by an increased DNR pumping rate, which indicates that PMA-induced phosphorylation does not activate P-gp.

Influence of PMA on the Intracellular pH

It has been reported that PMA increases the intracellular pH by stimulating the Na^+/H^+ antiporter [26, 27]. Since DNR transmembrane transport is pH-dependent, a PMA-induced increase in cytosolic pH would result in lower cellular DNR accumulation [23, 28]. By measuring the intracellular pH with the pH probe BCECF, we found that after exposure to 100 nM PMA (15–25 min) the pH changed approximately +0.02 pH unit, both in the wild-type and resistant cells. Although the change in pH was not significant ($p > 0.1$), such a change would result in a decrease in DNR accumulation of 4%, according to the Henderson-Hasselbach equation [28]. Therefore, an increase in the intracellular pH could be only partially responsible for the effect of PMA on DNR accumulation.

Concentration Dependency of Vp

To examine the possibility that PMA decreased the capacity of Vp to inhibit P-gp, a concentration-response experiment was performed. Figure 5 shows the relative effect of the inhibition of P-gp on [^{3}H]DNR accumulation in KB-V1 cells at increasing concentrations of Vp in the presence and absence of 100 nM PMA. The concentrations needed for

TABLE 1. Effect of PMA on cellular [^{3}H]VP-16 accumulation

	[^{3}H]VP-16 (pmol/10^6 cells)		
	without PMA	with PMA	[^{3}H]VP-16 (% control with PMA)
KB-3-1	2.00 ± 0.50	1.25 ± 0.30	65 ± 6
KB-V1	1.00 ± 0.50	0.40 ± 0.25	40 ± 10
SW1573	2.35 ± 0.85	1.50 ± 0.55	65 ± 5
S1(1.1)	1.70 ± 0.80	0.90 ± 0.45	55 ± 6

The effect of 300 nM PMA on [^{3}H]VP-16 accumulation measured in the wild-type cells KB-3-1 and SW1573 and the MDR cells KB-V1 and S1(1.1) is given. Cells were incubated in medium containing 0.25 μM [^{3}H]VP-16 for 60 min and washed, and the cellular [^{3}H]VP-16 content was measured. Data are given as the mean ± SEM of four independent experiments.

50% inhibition (IC_{50} values) were 35 ± 10 μM without PMA and 45 ± 15 μM with PMA. The decrease in [^{3}H]DNR accumulation caused by PMA was similar at all Vp concentrations (22 ± 7%). This experiment shows that the PMA effect remains at high Vp concentrations, indicating that the effect of PMA on [^{3}H]DNR accumulation is not mediated via P-gp.

[^{3}H]VP-16 Accumulation

To exclude the possibility that nonmeasurable changes in cellular pH disturbed the outcome of the experiments and to investigate whether the results found for DNR would also be found with other substrates, VP-16 and Cal-AM were included in this study. Cellular VP-16 and Cal-AM accumulation did not depend on the extracellular pH (data not shown). Table 1 shows that a lower [^{3}H]VP-16 accumulation was found with PMA compared with the control without PMA. This decrease was found both in the wild-type and resistant cells. Comparing the wild-type and MDR cells, PMA induced a larger relative decrease in [^{3}H]VP-16 accumulation in the MDR cells. As with DNR, these data show a reduced [^{3}H]VP-16 drug accumulation independent of the phosphorylation state of P-gp but dependent on the action of PMA. In contrast to the results found with DNR, we now observed a larger relative decrease in [^{3}H]VP-16 accumulation in the MDR cells than in the wild-type cells. This may be due to increased activity of P-gp. However, from these results it is not clear whether this is due to stimulation of P-gp activity or to an amplification by P-gp of the same effect that is found in wild-type cells.

Cellular Cal-AM Accumulation

Cal-AM was used as a third substrate to study the effects of PMA on P-gp activity. Figure 6 shows two traces of the Cal-AM accumulation with MDR KB-8-5 and wild-type KB-3-1 cells, monitored in time. The P-gp substrate Cal-AM (nonfluorescent) entered the cells and was converted intracellularly to calcein (fluorescent), which is not a P-gp substrate. As can be seen in Fig. 6, calcein production was constant in time, and production was linear with the extracellular Cal-AM concentration from 0.1 μM to 1 μM (data not shown). This shows that the Cal-AM net influx rate and Cal-AM hydrolysis were in steady state. From this we may conclude that intracellular calcein production is proportional to the intracellular Cal-AM concentration.

By measuring the rates of calcein production and correcting for the background hydrolysis, an approximate

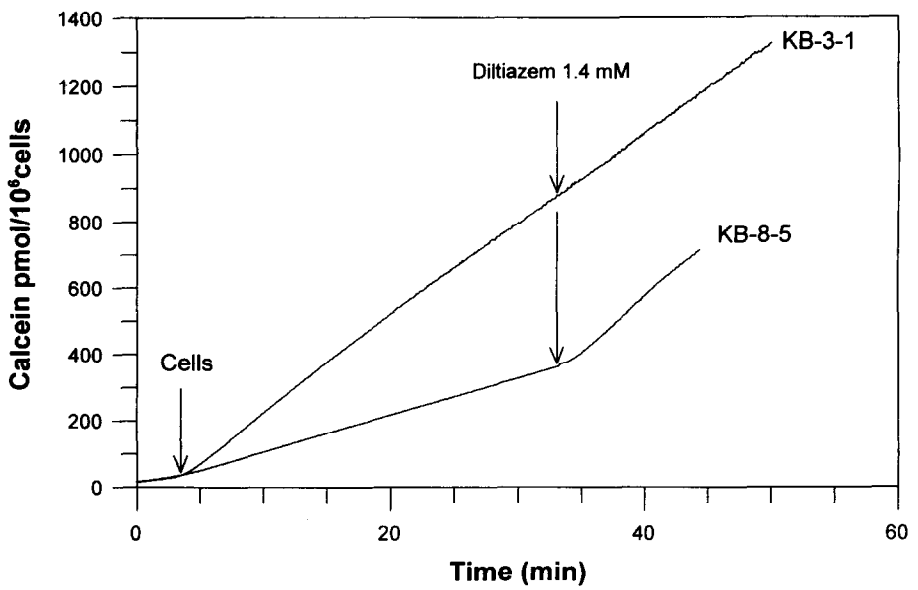

FIG. 6. The cellular calcein production of P-gp-expressing KB-8-5 and wild-type KB-3-1 cells in the presence of 0.5 μM Cal-AM. After a constant background hydrolysis was obtained, 0.1 × 10^6 cells/mL were added as indicated. The P-gp inhibitor diltiazem (1.4 mM) was also added as indicated. The amount of calcein produced was calculated from a standard curve made with calcein. Representative traces are shown.

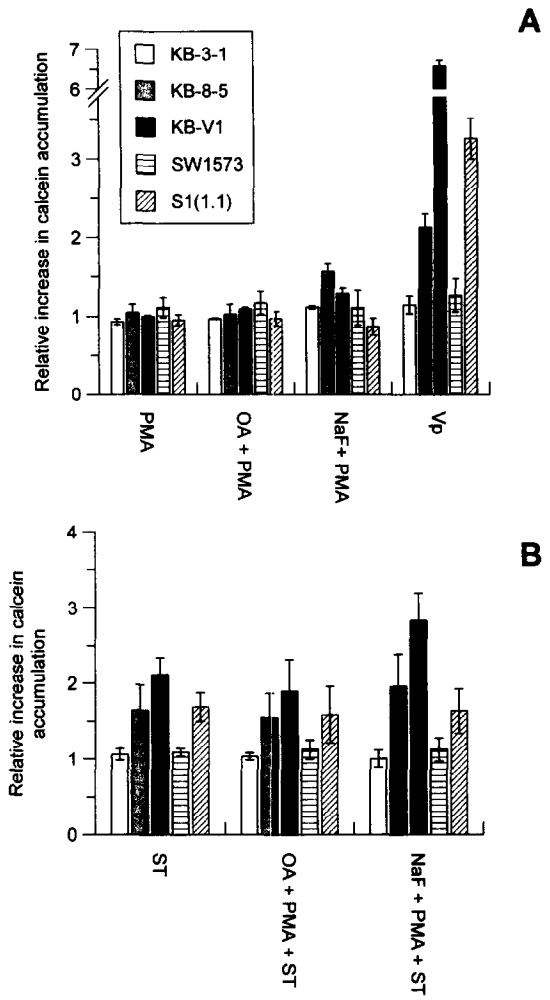

FIG. 7. The influence of different phosphorylation modulators on Cal-AM accumulation is shown in the MDR cells KB-8-5, KB-V1, and S1(1.1) and in the wild-type cells KB-3-1 and SW1573. A, the influence of PMA (100 nM), PMA with OA (200 nM), PMA with NaF (3.5 mM), and Vp (50 μM) on a 30-min Cal-AM accumulation. B, the effect of PMA (100 nM) in combination with OA (200 nM) or NaF (3.5 mM) on the inhibition of P-gp by ST (200 nM). The accumulation when no modulator was added was set at 1. Data are given as the mean ± SEM of three independent experiments.

3-fold increase in intracellular Cal-AM concentration was calculated when P-gp was inhibited with diltiazem in KB-8-5 cells. As can be seen in Fig. 6, diltiazem had no effect on wild-type cells. Using other P-gp inhibitors, such as PSC833 or Vp, or depleting the cells of ATP with sodium azide resulted in the same increase in calcein production (data not shown). Similar results were obtained for the MDR cells KB-V1 and S1(1.1) (not shown).

Figure 7 shows the effects of different phosphorylation modulators and combinations on Cal-AM transport using the MDR cells KB-8-5, KB-V1, and S1(1.1) and the wild-type cells KB-3-1 and SW1573. In this figure, the effect of P-gp activity is expressed as the ratio of the cellular calcein accumulation rate with modulator to that without modulator. Figure 7A shows that the PKC activator PMA or a combination of PMA with the PP1/PP2A inhibitor OA did not decrease Cal-AM accumulation either in the MDR cells or in the wild-type cells. Similarly, using the phosphatase inhibitor NaF instead of OA to inhibit cellular phosphatase activity did not lead to a stimulation of P-gp activity. Incubating the cells with dibutyryl cyclic AMP (dibutyryl cAMP) to stimulate PKA, which could lead to phosphorylation and a possible regulation of P-gp [16], did not lead to a change in Cal-AM accumulation in either the MDR or wild-type cells. Vp (50 μM) increased the Cal-AM accumulation in the MDR cells, demonstrating that P-gp was functionally present.

As reported by others, the kinase inhibitor ST inhibited P-gp activity and increased Cal-AM accumulation (Fig. 7B). To distinguish between a direct inhibition of P-gp by ST or an inhibition of ST via its kinase inhibitor activity, we examined whether inhibition of the dephosphorylation of P-gp decreased the degree of inhibition of ST. Before the additions of ST and Cal-AM, the cells were incubated for 15 min with both OA and PMA or with NaF and PMA to drive P-gp into its state of maximal phosphorylation. Figure 7B shows that the inhibitory effect of ST in the MDR cells was not decreased by the phosphatase inhibitors. In the control experiments with wild-type cells, no effect of either phosphorylation modulator on Cal-AM accumulation was observed, indicating that if changes in calcein production in the MDR cells did occur, these would be due to a change in P-gp activity.

DISCUSSION

The hypothesis that a P-gp-expressing cell can rapidly respond to a toxic assault by immediately increasing its P-gp pumping activity by changing its phosphorylation state, as was postulated in the literature [5], is attractive, since via induction at a transcriptional level [29] the response would be much slower. However, we could not find evidence for such an immediate response. Since we observed a PMA-induced decrease in [^{3}H]DNR and [^{3}H]VP-16 accumulation in wild-type cells, this made it difficult to conclude that the PMA effects were P-gp dependent. In principle, the decreased accumulation in wild-type cells is in accordance with the findings of Chambers *et al.* [5–7], who reported that PMA was effective in decreasing vinblastine accumulation of KB-V1 cells when P-gp was inhibited by Vp. However, we found a similar relative effect of PMA on the DNR accumulation in MDR cells, whether Vp was present or not. In contrast with previous reports [5, 11, 12] that suggested that phorbol esters had only a marked effect on the drug accumulation of MDR cells, our results suggest that phorbol esters have similar results whether P-gp is actively present or not. Chambers *et al* [5–7]. showed that PMA increases the phosphorylation of P-gp in KB-V1 cells. However, their results did not discriminate between a direct effect of PMA on P-gp or a P-gp-independent mechanism that resulted in a decreased drug accumulation. Although the effect of PMA on [^{3}H]DNR accumulation

could perhaps be partially explained by a change in cellular pH, comparable results were obtained for [^{3}H]VP-16, for which cellular accumulation is pH-independent. Furthermore, the decreased dip depth in our flow-through experiment shows that P-gp is not activated after addition of PMA and that the effect of PMA on DNR accumulation lasted for several hours.

The only result that could be explained by an increased pumping rate was the larger decrease in [^{3}H]VP-16 accumulation in MDR cells compared with wild-type cells. However, it is possible that the decrease in MDR cells is simply larger because the P-gp amplifies the same effect that is observed in wild-type cells. For example, a decreased passive diffusion across the membrane may be responsible for making the P-gp more effective instead of activating it.

Since we found that the accumulation of Cal-AM in wild-type cells was not affected by modulators of PKC or by the pH, Cal-AM seemed to be a better substrate for studying the possible regulation of P-gp transport activity. We did not observe any activation of Cal-AM transport in any of the MDR cells. In addition, dibutyryl cAMP and NaF, compounds that increase PKA activity and decrease phosphatase activity, respectively, did not stimulate P-gp activity. From these results, we conclude that both PKC and PKA do not directly influence P-gp-mediated drug-transport activity. This conclusion is in accordance with Smith and Zilfou [30], who reported that P-gp-mediated vinblastine transport was independent of protein kinases. Furthermore, our results complement those of Germann *et al.* [17] and Goodfellow *et al.* [31], who used a genetically modified form of P-gp that could not be phosphorylated at eight of the potential phosphorylation sites and showed that this nonphosphorylated form of P-gp still gave an MDR phenotype to cells and remained capable of Cal-AM transport. In comparison with those studies that used a mutated form of P-gp, our study goes further in that it provides evidence that Cal-AM transport mediated by P-gp is not influenced by the increased phosphorylation state of P-gp while excluding the possibility that PKC or proteins under the control of PKC directly influence P-gp activity. In addition, we show that ST inhibits Cal-AM transport in a protein phosphorylation-independent way, since the ST reversing effect on Cal-AM accumulation persisted when the dephosphorylation was inhibited. Such an inhibition was also suggested by Miyamoto *et al.* [13, 14] who showed that the degree of PKC inhibition and of P-gp inhibition of different ST derivatives did not correlate.

In conclusion, our data show that PMA induces a decrease in [^{3}H]DNR and [^{3}H]VP-16 accumulation in P-gp-expressing cells, which is not the result of stimulation of pumping by P-gp. For [^{3}H]DNR no increased effect of PMA was observed in the MDR cells compared with the wild-type cells, nor was an increase in dip depth found in the presence of PMA in the flow-through experiments. The absence of any effect on Cal-AM pumping further substantiates the notion that P-gp activity is not affected by PMA. We conclude that PMA-induced PKC-mediated phosphorylation of P-gp is not a general method of the cell to increase drug transport activity. Our results do not exclude the possibility that certain substrates may be sensitive to the phosphorylation state of P-gp. The mechanisms induced by PMA that cause the effects on DNR and [^{3}H]VP-16 accumulation are not yet understood. However, our experiments provide evidence that PKC or other PMA- and ST-sensitive proteins play a role. Since these mechanisms cause a large decrease in the cellular accumulation of these anticancer drugs, i.e. approximately 20% for DNR and 35% for VP-16, these mechanisms probably also play a role in the resistance of cells to anticancer drugs. Understanding of these mechanisms will give us more insight into the often multifactorial nature of MDR in cancer cells.

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