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PKC-independent Modulation of Multidrug Resistance in Cells with Mutant (V185) but not Wild-Type (G185) P-Glycoprotein by Bryostatin 1

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ABSTRACT. Bryostatin 1 is a new antitumor agent which modulates the enzyme activity of protein kinase C (PKC, phospholipid-Ca^{2+}-dependent ATP:protein transferase, EC 2.7.1.37). Several reports have suggested that the pumping activity of the multidrug resistance gene 1 (MDR1)-encoded multidrug transporter P-glycoprotein (PGP) is enhanced by a PKC-mediated phosphorylation. It was shown here that bryostatin 1 was a potent modulator of multidrug resistance in two cell lines over-expressing a mutant MDR1-encoded PGP, namely KB-C1 cells and HeLa cells transfected with an MDR1-V185 construct (HeLa-MDR1-V185) in which glycine at position 185 (G185) was substituted for valine (V185). Bryostatin 1 is not able to reverse the resistance of cells over-expressing the wild-type form (G185) of PGP, namely CCRF-ADR5000 cells and HeLa cells transfected with a MDR1-G185 construct (HeLa-MDR1-G185). Treatment of HeLa-MDR1-V185 cells with bryostatin 1 was accompanied by an increase in the intracellular accumulation of rhodamine 123, whereas no such effect could be observed in HeLa-MDR1-G185 cells. HeLa-MDR1-V185 cells expressed the PKC isoforms α, δ and ζ. Down-modulation of PKC α and δ by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) did not affect the drug accumulation by bryostatin 1. Bryostatin 1 depleted PKC α completely and PKC δ partially. In HeLa-MDR1-V185 cells, short-term exposure to bryostatin 1, which led to a PKC activation, was as efficient in modulating the pumping activity of PGP as long-term exposure leading to PKC depletion. Bryostatin 1 competed with azidopine for binding to PGP in cells expressing the MDR1-V185 and MDR1-G185 forms of PGP. It is concluded that bryostatin 1: i) interacts with both the mutated MDR1-V185 and the wild-type MDR1-G185; ii) reverses multidrug resistance and inhibits drug efflux only in PGP-V185 mutants; and iii) that this effect is not due to an interference of PKC with PGP. For gene therapy, it is important to reverse the specific resistance of a mutant in the presence of a wild-type transporter and vice versa. Our results show that it is possible to reverse a specific mutant PGP. BIOCHEM PHARMACOL **56**;7:861–869, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. multidrug resistance; P-glycoprotein; bryostatin 1; protein kinase C; rhodamine 123; reversal

Bryostatin 1, a macrocyclic lactone isolated from the marine bryozoan Bugula neritina [1], gained considerable attention as a unique modifier of biologic responses, showing a remarkable antitumor activity against a variety of murine tumors [2, 3]. Currently, it is undergoing phase 1 clinical evaluation as an anticancer drug [4]. Bryostatin exhibits a remarkable affinity for various isozymes of the PKC§ (phospholipid-Ca^{2+}-dependent ATP:protein transferase, EC 2.7.1.37) family [5]. In many respects, it mimics the activities of tumor-promoting phorbol esters. Like TPA, binding of bryostatin 1 activates PKC [6, 7]. Prolonged exposure of intact cells to bryostatin, just as to phorbol esters, leads to a translocation of PKC from the cytosol to the membrane and a depletion of the enzyme, most likely due to proteolytic degradation [8–10]. Although there are differences in the range of effects elicited by bryostatin 1 or TPA [11, 12], it is believed that enzymes of the PKC family are the preferred targets of both compounds.

The MDR1-encoded PGP or multidrug transporter catalyzes an ATP-dependent efflux of several antitumor agents. Over-expression of MDR1 leads to multidrug resistance [13, 14]. A large body of observations suggests that a change in the phosphorylation state of PGP might affect its ability to transport cytotoxic drugs [15]. The MDR phenotype is frequently associated with elevated levels of PKC [16–19]. Treatment of cells with the PKC activator TPA has been shown to lead to an increased phosphorylation of the multidrug transporter and at the same time to a reduced

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§ *Abbreviations*: HeLa-WT, drug-sensitive HeLa wild-type cells; HeLa-MDR1-G185, multidrug-resistant HeLa cells transfected with a wild-type MDR1 gene; HeLa-MDR1-V185, multidrug-resistant HeLa cells transfected with a mutant MDR1 gene (valine instead of glycine in position 185); MDR, multidrug resistance; MDR1, multidrug resistance gene 1; PGP, P-glycoprotein; PKC, protein kinase C; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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drug accumulation [14–16]. PKC comprises a family of 11 closely related isoenzymes. The α isoform seems to be of importance for multidrug resistance as over-expression of PKCα increases MDR in cells expressing the PGP [20,21]. Expression of antisense cDNA for PKCα reduces drug resistance [22]. On the other hand, it has been reported that reversal of MDR by PKC inhibitors is elicited by their direct interaction with PGP and not due to inhibition of PGP-phosphorylation by PKC [23–27]. PGP contains five phosphorylation sites for PKC and other kinases. Replacement of these serines by nonphosphorylatable alanines does not have any effect on the activity of PGP [28].

Because there are contradictory reports on the role of PKC in the modulation of PGP, and bryostatin 1 acts as a selective modulator of PKC, it appeared of interest to find out whether bryostatin 1, in addition to having antitumor activity, is also capable of modulating MDR1-mediated MDR. Recently Scala *et al.* reported that bryostatin 1 reduces the phosphorylation of PGP, but does not reverse resistance of multidrug-resistant MCF-7 cells [29]. As we describe here, we found that bryostatin 1 reverses MDR of human KB-C1 cells. We investigated whether this discrepancy could be explained. We found that it was not PKC that was responsible for this difference, but rather a mutation at position 185 of PGP. As it is difficult to crystallize large membrane proteins no detailed three-dimensional structure of this family of transporters is currently available nor is the exact function of PGP known at present. Therefore, the effects of mutations may help explain the mechanism of PGP action.

MATERIALS AND METHODS

Drugs

Vinblastine, colchicine and rhodamine 123 were purchased from Sigma. Bryostatin 1 was kindly provided by Dr. G. R. Pettit, Cancer Research Institute.

Cell Culture

Human KB-3–1 and KB-C1 cells [30] (from Dr. M. M. Gottesman) were grown in Dulbecco's modified Eagle's medium (4.5 g/L of glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/mL of penicillin and 50 μg/mL of streptomycin in 5% CO_2. One microgram of colchicine/mL was added to the medium of the multidrug-resistant KB-C1 cells. CCRF-CEM and CCRF-ADR5000 cells [31] were a gift from Dr. V. Gekeler, Byk–Gulden, and were grown in RPMI 1640 medium, supplemented as described for KB cells. CCRF-ADR5000 cells were grown in the presence of 5 μg of adriamycin/mL, except at the time of experiments.

The two multidrug-resistant MDR1-over-expressing cell lines were obtained by transfection of human HeLa S3 (HeLa-WT) cells with an MDR1 wild-type gene construct (HeLa-MDR1-G185) or the same construct with a mutation in codon 185 (HeLa-MDR1-V185, gly to val; kindly provided by Dr. M. M. Gottesman) [32]. Single cells of the transfectants were grown and MDR1-over-expressing clones were selected. The expression of MDR1 mRNA was detected by reverse transcription polymerase chain reaction (PCR) and is shown in the Results section. The wild-type (CCRF-ADR5000 and HeLa-MDR1-G185) and mutant genes (KB-C1 and HeLa-MDR1-V185) were determined to be such by sequencing (Table 1). All three HeLa cell lines were grown in RPMI 1640 supplemented as described for KB cells. One hundred nM vinblastine and 240 ng of colchicine/mL were added to the stock cultures of HeLa-MDR1-G185 cells and HeLa-MDR1-V185 cells, respectively, every other week.

TABLE 1. Nucleotide sequences of the MDRI genes with the corresponding amino acid sequences in CCRF-ADR 5000, HeLa-MDR1-G185, KB-C1 and HeLa-MDR1-V185 cells covering the proximity of amino acid position 185 of PGP

			185		
CCRF-ADR5000	AAT Asn	GAA Glu	**GGA** ***Gly***	ATT Ile	GGT GLY
HeLa-MDR1-G185	AAT Asn	GAA Glu	**GGA** ***Gly***	ATT Ile	GGT Gly
KB-C1	ATT Asn	GAA Glu	**GTT** ***Val***	ATT Ile	GGT Gly
HeLa-MDR1-V185	AAT Asn	GAA Glu	**GTT** ***Val***	ATT Ile	GGT Gly

DNA sequencing was performed as described in Materials and Methods.

To assay the inhibition of cell proliferation, cells were seeded in 96-well plates. Two hours after plating of the cells, drugs were added as indicated in the figures, followed by continuous incubation for 72 hr. Subsequently, cell proliferation was detected by the sulforhodamine B assay [33].

Accumulation of Rhodamine 123

Logarithmically growing cells were trypsinized, washed with PBS and resuspended in 1 mL (5.10^5 cells/mL) of Dulbecco's modified Eagle's medium without serum, supplemented with 20 mM 3-*N*-morpholinopropanesulfonic acid. The cells were incubated with the drugs at 37° for the times indicated in the figures. After addition of 60 μL (5 μg/mL) of rhodamine 123 per mL cell suspension, fluorescence (excitation wavelength at 488 nm) was observed by a flow cytometer (FACStar, Becton Dickinson) at the times indicated in the figures. Emission was observed with a 530/30-nm filter, and fluorescence intensity was expressed as the mean of 5000 cells gated by forward and side-scattered light to measure only viable and single cells [34].

Western Blot Analysis

Polyclonal antibodies directed against amino acids 313–326 of rat PKC α generated in rabbits were from Life Technologies. PKC ζ polyclonal antibodies (Life Technologies) were obtained against amino acids 577–592 of rat brain PKC ζ. Polyclonal antibodies directed against amino acids 657–676 of human PKC δ were obtained from Santa Cruz Biotechnology Inc. 1–2.10^8 HeLa-MDR1-V185 cells were washed with ice-cold PBS, resuspended in 50 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 1 mM EGTA, 25 μg of aprotinin/mL, 25 μg of leupeptin/mL and lysed by repeated freezing and thawing. After centrifugation at 280,000 *g* at 4° for 10 min, the supernatant (representing the cytosolic fraction) was collected. The remaining pellet was resuspended in the buffer described above, containing 1% Triton X-100, and incubated at 4° for 30 min and subsequently centrifuged at 280,000 *g* at 4° for 10 min, the supernatant representing the particulate fraction. The protein content of each fraction was determined by a BCA assay (Pierce). After separation on a 4–15% polyacrylamide gel containing SDS, the protein was transferred electrophoretically onto a membrane (Immobilon-P, Millipore) using a mini transblot cell (Bio-Rad Laboratories) in Towbin-buffer (25 mM Tris-HCl, pH 8.3, 193 mM glycine, 0.1% SDS, 20% methanol) at 150 mA for 1.5 hr. Membranes were blocked with 1% BSA and subsequently incubated with the individual antibodies at room temperature for 2 hr. The PKC α and ζ antibodies were diluted 1:100, the final concentration of the δ antibody being 1 μg/mL. After washing with TBS containing 0.1% Tween-20, the membranes were incubated at room temperature with a peroxidase-linked secondary antibody for 1 hr, treated with an enhanced chemoluminescence Western blotting detection kit (Amersham) and exposed to an enhanced chemoluminescence Hyperfilm (Amersham). To confirm the different PKC isoenzyme-specific bands, PKC from rat brain or PKC isoenzyme standards were used. In addition, competition with blocking peptides was performed according to the manufacturer's instructions.

Photoaffinity Labeling with [^{3}H]Azidopine

Membranes from HeLa-WT, HeLa-MDR1-V185, CCRF-CEM and CCRF-ADR5000 cells were prepared according to Hamada and Tsuruo [35]. The protein concentration was determined using an assay kit from Pierce. Under dim light, 30 μg of protein were incubated with 0.7 μCi of [^{3}H]azidopine (49 Ci/mmol, Amersham) in the presence or absence of bryostatin 1 in a final volume of 35 μL of phosphate buffer (40 mM, pH 7.4) at room temperature for 60 min. Subsequently, the reaction mixture was placed on ice and irradiated with a UV lamp (CAMAG, Merck) at a distance of 8 cm for 20 min. Samples were separated on a SDS-polyacrylamide gel (4–15%) and exposed to an x-ray film.

MDR1 mRNA Levels and Sequencing

Total RNA was isolated using RNAzol (Biotexs Laboratories Inc.). cDNA was synthesized with 5 μg of total cellular RNA and 5 pmol/μL of hexanucleotide random primers (Boehringer Mannheim) at 37° for 1 hr in a volume of 50 μL, containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 100 μg/mL of BSA, 20 mM β-mercaptoethanol, 1 mM each deoxynucleotide, 1.25 units of RNasin (Promega) and 120 units of MMLV reverse transcriptase (Promega). Amplification of MDR1 was carried out by PCR using cDNA corresponding to 10 ng of total RNA in a volume of 50 μL, containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.2 mM deoxynucleotides each, 1.5 mM $MgCl_2$ and 3 units of Taq-polymerase (Promega). The primers for MDR1 and β-microglobulin were exactly as described by Noonan *et al.* [36]. Thirty cycles were performed, with a denaturation temperature of 94° for 35 sec, an annealing temperature of 57° for 30 sec and an extension temperature of 72° for 1 min. The reaction products were separated on a 10% polyacrylamide gel and stained with ethidium bromide for determination of the mRNA levels. For sequencing, the cDNA was amplified (30 cycles, 94° for 35 sec, 57° for 30 sec, 72° for 1 min) with the primers: CTGGTTTGATGTGCACGATGTT and GGCCAAAATCACAAGGGTTAGC, covering the sequence (178 bases) around the amino acid position gly185/val185. Sequencing was performed by a nonisotopic DNA sequencing kit (Clontech Laboratories Inc.) using the biotinylated primers described above. The sequences were detected by an enhanced chemoluminescence kit from UBS following the manufacturers instructions.

RESULTS

Bryostatin 1 Reversal of Resistance in Cells Expressing Mutant PGP

Scala *et al.* reported that bryostatin 1 does not reverse MDR in MCF-7 cells [29]. In contrast to this, we found bryostatin 1 to be a potent agent in reversing the resistance of MDR1-over-expressing KB-C1 cells to vinblastine as well as to colchicine (Fig. 1A, 1B). Although 1 μM bryostatin 1 did not reverse MDR1-mediated resistance to vinblastine and colchicine in CCRF-ADR5000 cells (Fig. 1, C and D), an observation which is in agreement with that of Scala *et al.* [29], we found that such a reversal could be achieved by 500 nM in KB-C1 cells (Fig. 1A and 1B). If administered as a single agent, the growth-inhibitory effect of bryostatin 1 was not significant at 500 nM (approximately 10%) and did not exceed 20% at 1 μM (data not shown).

It has been reported that KB-C1 cells harbor a mutated MDR1 gene in which glycine at position 185 is replaced by valine [37]. In order to determine whether CCRF-ADR5000 cells contain a wild-type and to confirm the presence of a mutation in position 185 of PGP in KB-C1 cells, we sequenced the cDNAs. The results confirmed the sequence difference between the two cell lines (Table 1).

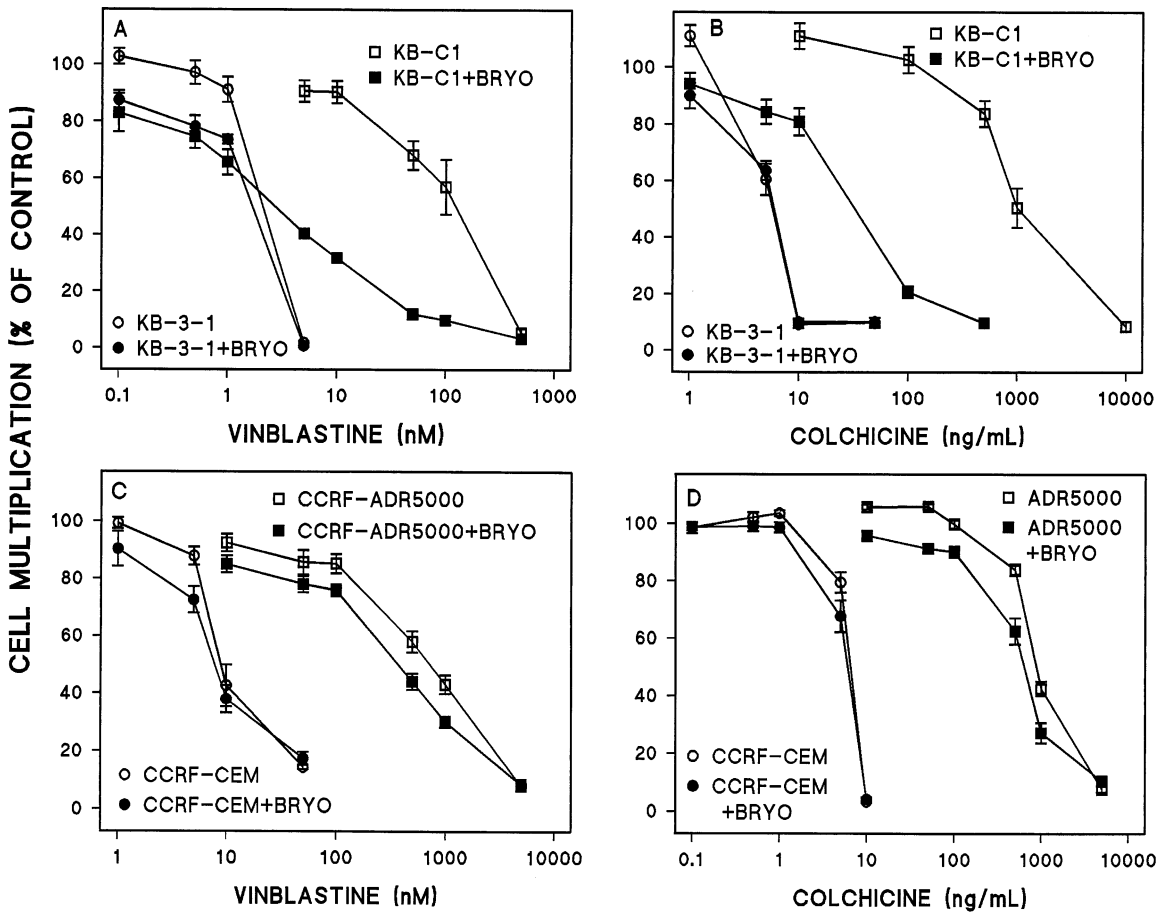

FIG. 1. Reversal of MDR by bryostatin 1 in KB and CCRF cells. Effects of vinblastine and colchicine alone or in combination with bryostatin 1 on drug-sensitive KB-3–1, multidrug resistant KB-C1 cells (A, B) and sensitive CCRF-CEM and multidrug-resistant CCRF-ADR5000 cells (C, D). Cell proliferation was determined by the sulforhodmamine B assay as described in Materials and Methods. In combination with reversing drugs, KB-3–1 and KB-C1 cells were treated with 500 nM, and CCRF-CEM and CCRF-ADR5000 cells with 1 μM bryostatin 1. Data represent the means of at least three independent experiments (±SEM), in which duplicate determinations were taken within each experiment.

In order to prove that it is the mutated PGP in KB-C1 that is responsible for reversal of MDR by bryostatin, we stably transfected drug-sensitive human HeLa-WT cells with MDR1-G185 and MDR1-V185, respectively. Figure 2 shows that the stably transfected clones exhibited resistance to vinblastine and colchicine. The expression levels of the MDR1 mRNA in the cell lines are shown in Fig. 3. The presence of the wild-type and mutated genes was established by sequencing (Table 1). Pretreatment of the sensitive, parental line (HeLa-WT) with bryostatin 1 had only a marginal effect on the antiproliferative activities of vinblastine and colchicine. The resistance of HeLa-MDR1-G185 transfectants to vinblastine or colchicine treatment was also not affected by the addition of bryostatin (Fig. 2, C and D). However, pretreatment of the MDR1-V185-transfected cells with 500 nM of bryostatin 1 for 24 hr sensitized the cells to both vinblastine and colchicine and thus led to a reversal of drug sensitivity (Fig. 2A, B). These data clearly show that reversal of MDR1-mediated resistance by bryostatin 1 could be accomplished only in cells harboring a mutant (V185) MDR1 gene.

Inhibition of Drug Efflux by Bryostatin 1

In order to investigate whether bryostatin 1 inhibits the pumping activity of the mutant MDR1-V185-encoded PGP, the intracellular accumulation of rhodamine 123 was determined. The fluorescent dye rhodamine 123 has been shown to be an excellent substrate of the multidrug transporter [34]. In Fig. 4, the time course of rhodamine 123 accumulation is shown in three different HeLa cell lines, namely in HeLa-WT, HeLa-MDR1-G185 and HeLa-MDR1-V185, respectively. In the drug-sensitive HeLa-WT cells, there was considerable accumulation of rhodamine 123, while due to the pumping activity of PGP in both MDR1-over-expressing subclones, the rhodamine levels remained low. However, in the MDR1 mutant expressing cell line HeLa-MDR1-V185, bryostatin led to an increase in the accumulation of rhodamine (Fig. 4A). In the MDR1 wild-type expressing the Hela-MDR1-G185 clone, the intracellular concentration of rhodamine 123 did not increase significantly following pretreatment with bryostatin 1 (Fig. 4B). This was in agreement with the MDR reversing

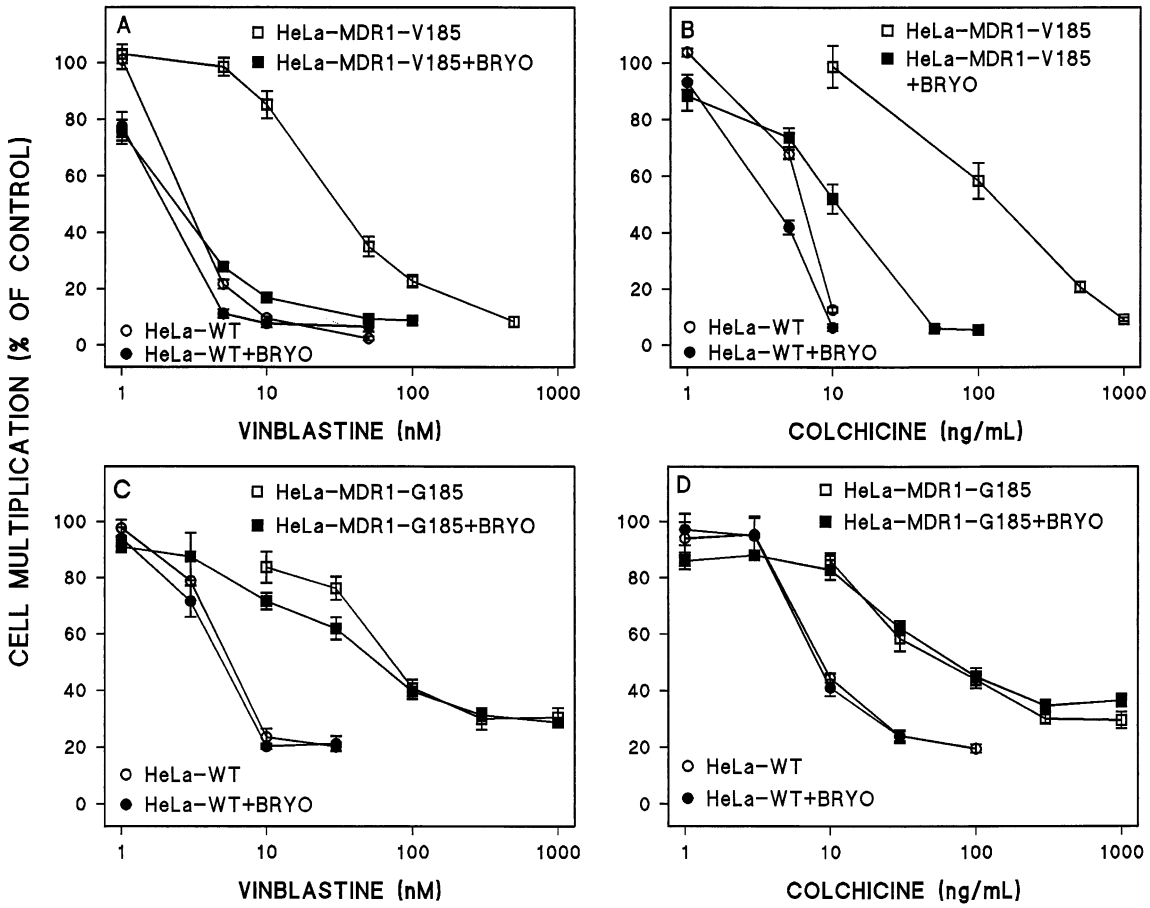

FIG. 2. Reversal of MDR in MDR1 transfectants by bryostatin 1. Effects of vinblastine and colchicine, either alone or in combination with bryostatin 1 (500 nM) on drug-sensitive HeLa-WT and multidrug-resistant HeLa-MDR1-G185 and HeLa-MDR1-V185 cells. Cell proliferation was determined by the sulforhodamine B assay as described in Materials and Methods. Data represent the means of at least three independent experiments (±SEM), in which duplicate determinations were taken within each experiment.

capability of bryostatin 1 in the MDR1-transfected cell lines (Fig. 2).

The Role of PKC in Reversal of Resistance

There are contradictory results on the role of PKC in modulation of MDR. A large number of reports suggest an involvement of PKC in PGP activity [14–22]. However, there are others that question the role of PKC in MDR1-mediated resistance [23–28]. In order to obtain some clarity in this matter, we investigated whether the difference in wild-type and mutant PGP in regard to reversal of resistance by bryostatin is related to PKC. As shown in Fig. 5, HeLa-MDR1-V185 cells expressed the PKC isoforms α, δ and ζ. Bryostatin 1 has been shown to interact with the diacylglycerol binding domain of PKC, the same region where phorbol esters are supposed to bind. As this region is missing in PKC ζ, only PKC α and δ remain as possible targets of bryostatin 1. Figure 5 shows that bryostatin indeed affected both the α and δ isoforms, while no effect was seen with PKC ζ. Exposure to 500 nM of bryostatin 1 caused a rapid translocation of PKC α from the cytosol (C) to the particulate (P) fraction, which is usually correlated to an activation of the enzyme. Prolonged exposure to bryostatin 1 led to a complete depletion of PKC α and almost no depletion of PKC δ. However, ζ was neither translocated nor depleted by bryostatin 1. Similar effects were obtained after exposure of HeLa-MDR1-V185 cells to the phorbol ester TPA: a 24-hr exposure to 1 μM TPA caused a complete depletion of PKC α and a significant reduction of δ (Fig. 5).

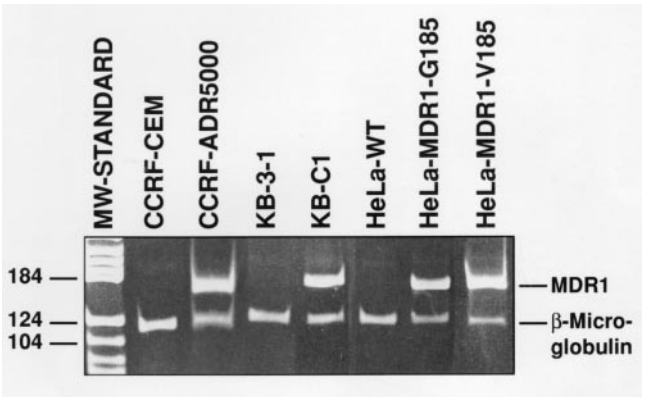

FIG. 3. MDR1 expression. The MDR1 mRNA levels of the indicated cell lines were determined by reverse-transcriptase PCR. β-microglobulin was employed as control for the amount of RNA used in the experiment.

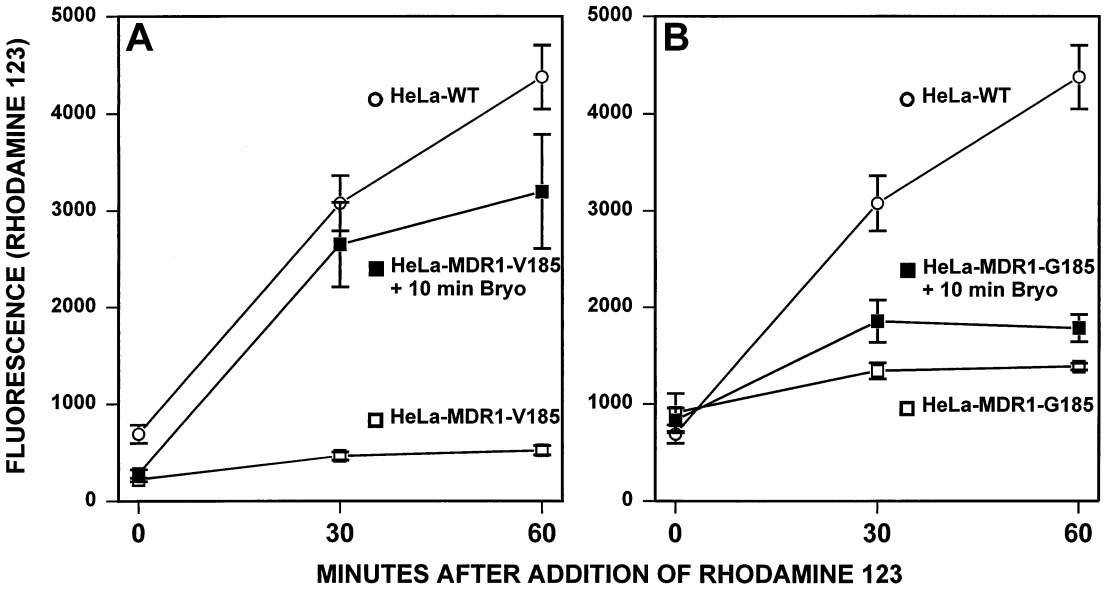

FIG. 4. Accumulation of rhodamine 123. The indicated cells were treated with 500 nM bryostatin 1 for 10 min. Subsequently, rhodamine 123 was added and the intracellular fluorescence assayed at the times indicated. Data represent the means of two independent experiments (±SEM), in which duplicate determinations were taken within each experiment.

If the modulation of multidrug resistance by bryostatin 1 is mediated by a PKC-dependent mechanism, activation or depletion of the bryostatin-responsive PKC isozymes should alter the potency of bryostatin 1 to act as a chemosensitizer in MDR1-V185-over-expressing cells. In order to determine a possible effect of PKC on the pumping activity of the V-185-mutated PGP, the intracellular accumulation of rhodamine 123 following treatment with TPA and bryostatin was investigated. If the reversal of resistance by bryostatin is caused by a depletion of PKC, down-modulation of PKC by long-term treatment with TPA should reduce the efflux activity of PGP and lead to an increase in the intracellular concentration of rhodamine 123. However, down-modulation of PKC by treatment of HeLa-MDR1-V185 cells with 1 μM TPA for 24 hr (Fig. 5) did not significantly reduce the pumping activity of V185-PGP (Fig. 6). Furthermore, pretreatment with 1 μM TPA for 24 hr did not affect the extent of intracellular accumulation of rhodamine elicited by bryostatin 1 (Fig. 6). These results demonstrate that a depletion of PKC α and δ by TPA did not affect the ability of bryostatin 1 to restore drug accumulation in MDR1-V185-transfected cells to levels of sensitive controls. In view of these findings, we conclude that bryostatin 1 modulates the mutant multidrug transporter by a PKC-independent mechanism.

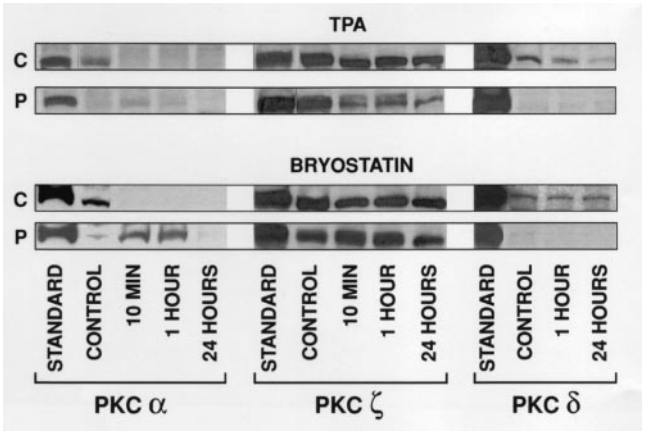

FIG. 5. Detection of PKC isoenzymes by Western blotting in the cytosolic (C) and particulate (P) fractions. HeLa-MDR1-V185 cells were treated with 1 μM TPA or 500 nM bryostatin 1 for the times indicated. Preparation of the cytosolic and particulate fractions, gel electrophoresis, transfer to membranes and incubation with the antibodies were performed as described in Materials and Methods. Following incubation with a horseradish peroxidase-conjugated secondary antibody, the membranes were treated with an enhanced chemoluminescence system and exposed to a Hyperfilm ECL.

Bryostatin 1 Competition with the Azidopine Binding Site of PGP

As the MDR-modulating effect of bryostatin 1 appears to be mediated by a PKC-independent mechanism, we wanted to find out whether bryostatin 1 interacts directly with PGP. This was determined by studying whether bryostatin 1 competes with azidopine for binding to the PGP extracts from membranes of cells expressing either wild-type (CCRF-ADR5000) or mutated PGP (HeLa-MDR1-V185). As shown in Fig. 7, a reduction in [^{3}H]azidopine binding to both wild-type and mutated PGP by bryostatin 1 could be observed. These results suggest that bryostatin 1 binds to the G185 as well as to the V185 form of PGP. The exact mechanism causing reversal of mutant PGP is not known at present. It is difficult to explain this mechanism, because the mutated form of PGP also has altered binding activities to cytotoxic drugs as well as to photolabeled compounds [37–39].

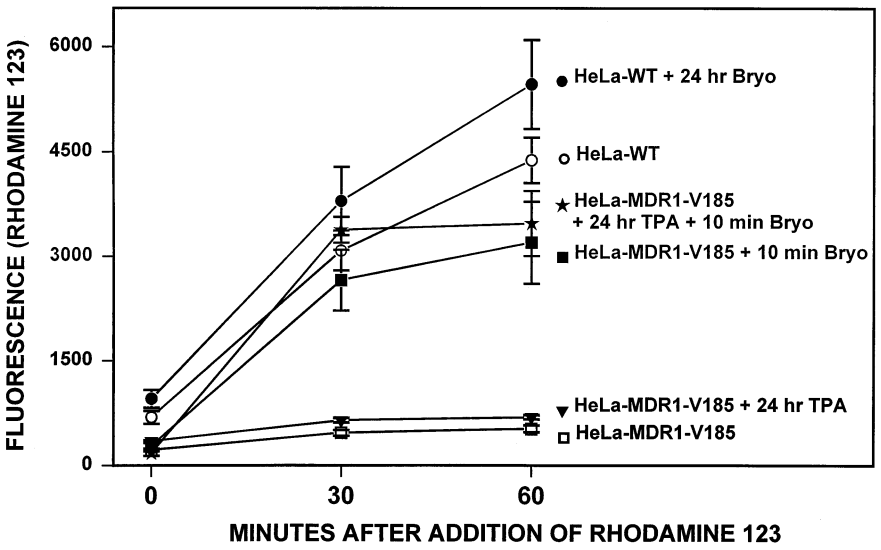

FIG. 6. Accumulation of rhodamine 123 following treatment with TPA and bryostatin 1. HeLa-WT and HeLa-MDR1-V185 cells were pretreated with 1 μM TPA or 500 nM bryostatin, or both compounds, for the times indicated. Following this incubation, rhodamine 123 was added and the intracellular fluorescence measured at the times indicated. Data represent the means of two independent experiments (±SEM), in which duplicate determinations were taken within each experiment.

DISCUSSION

The data presented here demonstrate that bryostatin 1 is able to reverse resistance to vinblastine and colchicine (and also to adriamycin, data not shown) in cells over-expressing mutant (V185) PGP, but not in the parental cells expressing the wild-type (G185) gene. The results of a number of investigations support the notion that phosphorylation of PGP by PKC increases resistance [14–22]. Thus, reduction of PKC activity should lead to an inhibition of the multidrug transporter. Previous studies have shown that a prolonged exposure to bryostatin 1 results in a depletion of several PKC isoforms [9–13]. It was intriguing, therefore, to postulate that the reversal of drug resistance in MDR1-expressing cells is caused by a depletion of PKC isoforms. The MDR1-V185-transfected HeLa cells employed here express the PKC isotypes α, δ and ζ. Chronic exposure to bryostatin 1 completely depleted cells of α and led to a partial reduction of δ, whereas ζ was not affected. This is consistent with the findings of other authors [9–13]. Similar effects are obtained by prolonged exposure of cells to the phorbol ester TPA. Thus, if bryostatin 1 affects the multidrug transporter by depleting PKC α or δ, TPA should mimic the effect of bryostatin 1 on drug accumulation in MDR1-V185-transfected cells. This, however, was not found to be the case (Fig. 6). Short-term exposure to bryostatin 1, which leads to an activation of PKC, is as efficient in eliciting drug accumulation as bryostatin treatment after preincubation with TPA leading to PKC depletion. These results also argue against the assumption that the inhibition of the pumping activity of PGP by bryostatin 1 is due to a depression of PGP phosphorylation. Our findings are consistent with a recent report by Scala *et al.* [29] demonstrating that prolonged exposure to bryostatin 1, which leads to a decreased phosphorylation of the multidrug transporter in MDR1-over-expressing MCF-7 human breast cancer cells, does not affect drug transport and drug resistance [29]. The authors did not indicate which form of PGP was expressed in the MCF-7 cells employed in their studies. The data of the present study are also in agreement with reports showing that PKC inhibitors reverse resistance by direct interaction and not by interference with the phosphorylation of PGP [23–27]. It has also been shown previously that a phosphorylation-defective PGP exhibits normal pumping activity [28].

In cells expressing MDR1-V185, bryostatin 1 is capable of inhibiting the efflux of the PGP-substrate rhodamine 123. Activation or depletion of PKC does not have any effect on the efflux of rhodamine 123 (Fig. 6). Wild-type PGP-expressing cells do not show alterations in the drug efflux following treatment with bryostatin 1 (Fig. 4). These results indicate that the reversal of drug resistance by bryostatin 1 is indeed caused by a blockade of the pumping activity of the MDR1-encoded multidrug transporter. The fact that the ability of bryostatin 1 to modulate MDR depends on the structure of PGP suggests a direct interaction of bryostatin with the PGP. This conclusion is supported by the competition of bryostatin 1 with azidopine for binding to PGP. However, bryostatin is binding to both the wild-type and mutated forms of PGP (Fig. 7).

Recently, it was demonstrated that cells expressing wild-type (G185) or mutant (V185) multidrug transporters exhibit differential sensitivities to drugs and modulators [37–39]. The inhibitors studied so far include quinidine, cyclosporine and verapamil. According to the data reported here, bryostatin 1 has to be added to this list. Due to the difficulty of crystallizing large membrane proteins, no detailed three-dimensional structure of this family of transporters is currently available and the exact function of PGP is not known at present. From results obtained with PGP mutants, such as those described here, the function of PGP may be finally established. Furthermore, the findings of the present study may be helpful in designing new MDR-reversing compounds. For gene therapy, it is important to reverse specifically the resistance of a mutant in the

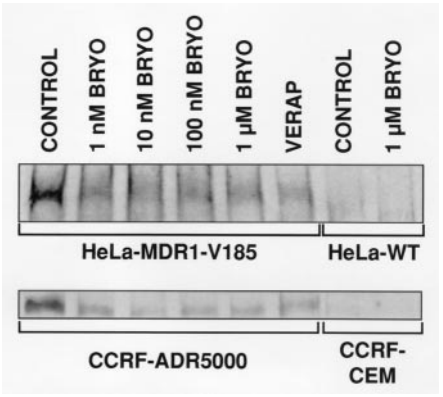

FIG. 7. Interaction of bryostatin 1 with PGP. Photoincorporation of [^{3}H]azidopine into PGP-enriched membranes of HeLa and CCRF cells was detected in the absence or presence of the indicated concentrations of bryostatin 1. Verapamil was used as a control. (BRYO = bryostatin 1, VERAP = verapamil).

presence of a wild-type transporter and vice versa [39–41]. This study describes that bryostatin 1 specifically reverses a mutant PGP.

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