Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Dexamethasone Regulation of P-Glycoprotein Activity in an Immortalized Rat Brain Endothelial Cell Line, GPNT

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Abstract: The blood–brain barrier (BBB) plays an important role in controlling the passage of molecules from the blood to the extracellular fluid environment of the brain. The multidrug efflux pump P-glycoprotein (P-gp) is highly expressed in the luminal membrane of brain capillary endothelial cells, thus forming a functional barrier to lipid-soluble drugs, notably, antitumor agents. It is of interest to develop an in vitro BBB model that stably expresses P-gp to investigate the mechanisms of regulation in expression and activity. The rat brain endothelial cell line, GPNT, was derived from a previously characterized rat brain endothelial cell line. A strong expression of P-gp was found in GPNT monocultures, whereas the multidrug resistance-associated pump Mrp1 was not expressed. The transendothelial permeability coefficient of the P-gp substrate vincristine across GPNT monolayers was close to the permeability coefficient of bovine brain endothelial cells cocultured with astrocytes, a previously documented in vitro BBB model. Furthermore, the P-gp blocker cyclosporin A induced a large increase in apical to basal permeability of vincristine. Thus, P-gp is highly functional in GPNT cells. A 1-h treatment of GPNT cells with dexamethasone resulted in decreased uptake of vincristine without any increase in P-gp expression. This effect could be mimicked by protein kinase C (PKC) activation and prevented by PKC inhibition, strongly suggesting that activation of P-gp function may involve a PKC-dependent pathway. These results document the GPNT cell line as a valuable in vitro model for studying drug transport and P-gp function at the BBB and suggest that activation of P-gp activity at the BBB might be considered in chemotherapeutic treatment of cancer patients. **Key Words:** Brain endothelial cells—P-glycoprotein—Blood–brain barrier—Dexamethasone.
J. Neurochem. **73,** 1954–1963 (1999).

In the CNS, endothelial cells forming the blood–brain barrier (BBB) are joined together by continuous tight junctions that prevent many substances from entering the brain. Although hydrophobic molecules such as nicotine and ethanol cross the BBB readily by passive diffusion, the brain microvasculature shows a highly restrictive permeability to hydrophobic antitumor agents (reviewed by Abbott and Romero, 1996). Studies on knock-out mice suggest a physiological role of the drug transporter, P-glycoprotein (P-gp), in preventing the entry of toxic substances, including chemotherapeutic agents, into the brain (Schinkel et al., 1995, 1997). This 150–180-kDa phosphoglycoprotein functions as an ATP-dependent drug efflux pump at the plasma membrane, maintaining low cytoplasmic drug concentrations (Gottesman and Pastan, 1993; Shapiro and Ling, 1995).

P-gp was originally identified as a protein overexpressed in cancer cells resistant to a diverse array of hydrophobic drugs (Gupta et al., 1988). In addition, functionally active P-gp has been shown to be present in nonmalignant tissues, notably, lung, intestine, and kidney epithelia, as well as brain and testis endothelia (Thiebaut et al., 1989; Cordon-Cardo et al., 1990; Drion et al., 1996). P-gp is encoded by a gene family comprising two *MDR* genes (*MDR1* and *MDR3*) in humans and three *mdr* genes (*mdr1a*, *mdr1b*, and *mdr2*) in rodents (Ng et al., 1989), although only the expression of human *MDR1* and rodent *mdr1a* and *mdr1b* appears to selectively confer multidrug resistance (MDR).

It is interesting that another recently described protein has been shown to be overexpressed in multidrug-resistant tumor cell lines, the MDR-associated protein (MRP) (Cole et al., 1992; Barrand et al., 1994). Furthermore, MRP has been found to be expressed at high levels in normal human lung, testis, and mononuclear blood cells (Cole et al., 1992) and at low levels in brain and other tissues (Zaman et al., 1993). The first human MRP de-

Received March 31, 1999; revised manuscript received July 22, 1999; accepted July 22, 1999.

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Abbreviations used: BBB, blood–brain barrier; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P-gp, P-glycoprotein; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

scribed is now called MRP1, and five homologues have been identified (Kool et al., 1999). In rodents, three Mrp-encoding genes have been identified, with *Mrp1* being involved like the human *MRP1* in MDR. In human and rat liver, *MRP2* and *Mrp2* are known as canalicular multispecific organic anion transporters (Keppler and Konig, 1997). Rat Mrp3 has been identified as an inducible transporter for the biliary excretion of organic anions (Kiuchi et al., 1998).

Previous in vitro studies have indicated that P-gp is present and functionally active in primary cultures of rat, porcine, and bovine cerebral endothelial cells (Greenwood, 1992; Huwyler et al., 1996; Fénart et al., 1998) and in immortalized cell lines (Tatsuta et al., 1992; El Hafny et al., 1997). However, down-regulation of P-gp expression has been documented in primary cultures of brain microvasculature endothelial cells compared with isolated brain capillaries (Barrand et al., 1995; Regina et al., 1998). By contrast with P-gp, MRP1 is not expressed in isolated human brain capillaries (Seetharaman et al., 1998), and Mrp1 is expressed at low levels in isolated rat brain capillaries (Regina et al., 1998). However, Mrp1 overexpression and activity are observed in cell cultures derived from these capillaries. Two other laboratories have described MRP activity in primary cultures of bovine primary brain endothelial cells and in an immortalized mouse brain capillary cell line (Huai-Yun et al., 1998; Kusuhara et al., 1998). Thus, a relevant in vitro model that closely resembles the BBB in vivo would be based on brain endothelial cells with a high P-gp expression and a low MRP1/Mrp1 expression.

Understanding the cellular factors that regulate the expression and/or the function of P-gp in brain endothelium should lead to the development of successful therapeutic strategies for overcoming MDR and restricted drug delivery into the brain. Glucocorticoids have been shown to reduce brain edema associated with malignant tumors and improve many related signs and symptoms, probably by a mechanism involving changes in BBB permeability (Grabb and Gilbert, 1995; Hoheisel et al., 1998). In addition, previous studies suggest that glucocorticoids may regulate P-gp activity. Indeed, transcriptional activation of MDR genes was demonstrated in hepatoma cells treated with dexamethasone, whereas dexamethasone produces the opposite effect in primary rat hepatocyte cultures (Fardel et al., 1993). Therefore, investigation of glucocorticoid effects on brain endothelial P-gp might be important in determining the effect of therapies combining glucocorticoids and anticancer drugs.

In this study, we have investigated the expression and activity levels of P-gp in an immortalized rat brain endothelial cell line, GPNT, derived from a previously characterized cell line, which retains morphological characteristics of primary brain endothelial cells and expression of specific brain endothelial markers and cell surface adhesion molecules (Greenwood et al., 1996). The present study reports the expression and activity of P-gp, but not Mrp1, in GPNT cells and shows that dexamethasone treatment increases P-gp efflux pump activity probably by a protein kinase C (PKC)-dependent mechanism.

MATERIALS AND METHODS

Chemicals

Fetal calf serum of American origin was obtained from GIBCO (Paisley, Scotland). [^{3}H]Colchicine was purchased from Dupont de Nemours (NEN Products, Les Ulis, France), and [^{3}H]vincristine was obtained from Amersham (Les Ulis, France). Cyclosporin A was a generous gift from Sandoz Pharma Ltd. (Basel, Switzerland). The C219 monoclonal anti-P-gp antibody was purchased from Valbiotech (Paris, France), the 6KQ polyclonal rabbit anti-MRP1 antiserum was kindly provided by Dr. M. Center (Kansas State University, Manhattan, KS, U.S.A.), and the monoclonal anti-ezrin antibody was purchased from Sigma (St. Quentin Fallavier, France). Electrophoresis reagents were from Amersham. All other reagents were from Sigma, unless otherwise specified.

Isolation of rat brain microvessels

Rat brain microvessels were isolated using a combination of the methods of Betz et al. (1979) and Abbott et al. (1992). Cortical gray matter free of meninges was incubated in 0.1% collagenase/dispase solution (Boehringer, Mannheim, Germany) for 2 h followed by density-dependent centrifugation through a 25% bovine serum albumin (BSA) solution. The pellet was resuspended in oxygen-saturated physiological buffer [147 m*M* NaCl, 4 m*M* KCl, 3 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 5 m*M* glucose, 15 m*M* HEPES (pH 7.4), and 1% (wt/vol) BSA] and then passed through a 1.2- $\times$ 1.5-cm column containing 0.25-mm-diameter washed glass beads. The isolated capillaries remained attached to the beads and were collected by repeated gentle agitation in physiological buffer and decantation. The final pellet was resuspended and washed several times in physiological buffer without BSA. An aliquot was taken for protein content measurement.

Cell culture

Rat brain endothelial cell primary culture. Brain capillary fragments were isolated from 2–3-month-old Sprague–Dawley rats according to the method of Abbott et al. (1992) with some modifications. Two or three rats were anesthetized by a combined-intraperitoneal injection of ketamine hydrochloride (50 mg/ml, 70 mg/kg; Parke-Davis, Morris Plains, NJ, U.S.A.) and diazepam (5 mg/ml, 7 mg/kg; Roche, Neuilly-Sur-Seine, France). After decapitation, brains were removed under sterile conditions. Cortical gray matter dissected free of meninges was incubated for 1 h in a collagenase/dispase solution plus DNase I and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone. The homogenate was then centrifuged in 25% BSA for removal of myelin, and the capillary fraction was incubated for 3 h in the collagenase/dispase solution to remove the capillary basement membrane. Finally, the capillaries were purified by filtration through a nylon mesh (pore size, 10 μm) and then plated out on 35-mm-diameter tissue culture dishes in culture medium [Ham's F-10 medium containing 16% plasma-derived bovine serum (First Link, U.K.), 2 m*M* glutamine, 80 μg/ml heparin, 50 μg/ml gentamicin, 75 μg/ml endothelial cell growth supplement, and additional supplements as listed by Abbott et al. (1992)]. The tissue culture dishes had been coated with type I collagen with further ammonia fixation. Cultures were maintained in humidified 5% CO_2/95% air at 37°C. After 3 days, contaminating pericytes surrounding endothelial cell colonies

were removed with a modified glass Pasteur pipette under the microscope. Thus, primary cultures of rat brain endothelial cells were estimated to be 100% pure by morphological criteria. When the endothelial cells had reached confluency, they were trypsinized briefly to remove them from the tissue culture dishes and plated out on collagen/fibronectin-coated plates. Experiments were performed on confluent 3- or 4-week-old cultures.

Rat brain endothelial cell lines. The GPNT cell line was obtained from a previously characterized rat brain endothelial cell line, GP8 (Greenwood et al., 1996). GP8 cells were retransfected with a selection plasmid containing the puromycin resistance gene. After repeated limiting dilution cloning of the parent line, a single clone, designated GPNT (from GP8 and the company NeuroTech S.A.), was selected using morphological criteria and retention of BBB characteristics. GPNT cells were maintained in α-minimal essential medium/Ham's F-10 (1:1 vol/vol) medium supplemented with 10% heat-inactivated fetal calf serum, 2 m*M* glutamine, 2 ng/ml basic fibroblast growth factor, 5 μg/ml transferrin, 5 μg/ml insulin, 5 ng/ml selenium, and 5 μg/ml puromycin in humidified 5% CO_2/95% air at 37°C. The phenotype of GPNT cells was found to be much more stable than that of the parental GP8 cell line.

RBE4 cell line. RBE4 cells (Roux et al., 1994) were plated on collagen I-coated plates and maintained in α-minimal essential medium/Ham's F-10 (1:1 vol/vol) medium supplemented with 10% heat-inactivated fetal calf serum, 2 m*M* glutamine, 300 μg/ml geneticin (G418), and 1 ng/ml basic fibroblast growth factor in humidified 5% CO_2/95% air at 37°C.

Western blotting

Protein fractions were prepared from cultured cells by solubilization in 20 m*M* Tris buffer (pH 7.4) containing 1 m*M* dithiothreitol, 1% Triton X-100, and a protease inhibitor cocktail (Boehringer). Cell lysates were agitated for 30 min on ice and then centrifuged for 5 min at 10,000 *g*. Protein concentrations of the supernatants were determined with a Bio-Rad protein assay. Protein samples were kept at −80°C until use. For immunoblotting, 2× Laemmli buffer was added to protein samples, which were then separated by electrophoresis on a 6% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane. P-gp was then identified by probing with the monoclonal C219 antibody (1 μg/ml), whereas the 6KQ polyclonal antibody (1:300 dilution) was used for Mrp1 analysis (Krishnamachary et al., 1994). Protein bands were detected by enhanced chemiluminescence (ECL; Amersham). After incubation with the anti-P-gp C219 and anti-Mrp1 6KQ antibodies, membranes were further incubated with an anti-ezrin monoclonal antibody (1 μg/ml) to verify equal protein loading in all lanes. The intensity of immunoreactive bands was quantitated by densitometry, and the ratios of P-gp-immunoreactive bands and ezrin-immunoreactive bands were calculated.

Functional analysis

Drug accumulation studies. Cellular uptake of the tritiated substrate [^{3}H]vincristine or [^{3}H]colchicine was measured as described previously (El Hafny et al., 1997). Confluent cells, grown in 24-well plates, were washed three times with phosphate-buffered saline (PBS) and preincubated for 30 min at 37°C in a shaking water bath with culture medium with or without the following inhibitors or competitive substrates: cyclosporin A (10 μM), verapamil (10 μM), colchicine (50 μM), or indomethacin (10 μM). Tritiated substrate (10 n*M*) was then added for 60 min. The plates were shaken during both preincubation and incubation periods to reduce the effect of the aqueous boundary layer on drug accumulation. The cells were rapidly washed three times with ice-cold PBS to eliminate the extracellular drug and then lysed in 500 μl of 0.1 *M* NaOH. The amount of tritiated drug retained in the cells was counted in Pico Fluor (Packard, France) by β-scintillation counting (Packard model 1900 TR). An aliquot of cell lysate was used in parallel to determine cellular protein concentration (Lowry et al., 1951). The intracellular colchicine or vincristine level was expressed in picomoles per milligram of protein.

To determine the effect of energy depletion on drug accumulation, cells were preincubated with culture medium with 10 m*M* deoxyglucose and 10 m*M* sodium azide to reduce ATP levels (Barrand et al., 1995).

Permeability of confluent GPNT monolayers. The permeability of GPNT cell monolayers was measured using a method adapted from those of Dehouck et al. (1992) and Romero et al. (1997). Cells were grown on collagen-fibronectin-coated polyester filters (Transwell-clear; 12 mm in diameter; pore size, 0.4 μm; Costar, Brumath, France) until confluence. Inserts containing confluent monolayers of GPNT cells were washed with PBS and then transferred to a well containing Dulbecco's modified Eagle's medium (DMEM) without phenol red supplemented with 10 m*M* HEPES and 10% fetal calf serum. DMEM with or without cyclosporin A (10 μM) was added for 30 min. The tracers [^{3}H]vincristine (Amersham; specific activity, 7.6 mCi/mmol; final concentration, 1 μCi/ml) and fluorescein (1 mg/ml) were added to the upper compartment, and after 5, 10, 20, 30, 40, and 60 min, inserts were sequentially transferred to another well. Incubations were performed at 37°C on a rotating shaker (100 rpm). Monolayers were then washed with PBS, and cells were solubilized in 0.1 *M* NaOH. The amounts of tracers in the lower compartment and within the cells were measured by β-scintillation counting (Packard model 1900 TR) for [^{3}H]vincristine and with a fluorescence multiwell plate reader (Wallac 1420 Victor) for fluorescein. Cleared volume was plotted against time, and the slopes of the curves, representing the clearance for each condition, were used to calculate the permeability coefficient (P_e) values of the endothelial monolayer: $1/PS = 1/m_e - 1/m_f$ and $P_e = PS/S$, where PS is the permeability–surface area product, S is the surface area of the filter (1.13 cm^2), and m_e and m_f are the slopes of the lines corresponding to endothelial cells on filters and to filters only, respectively.

Dexamethasone treatment

Drug sensitivity assay. The cytotoxicity of vincristine was evaluated in GPNT cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described by Romero et al. (1997). In brief, GPNT cells were plated in 96-well plates at a density of 5×10^3 cells per well with or without dexamethasone (1 μM, 3 days). Dexamethasone used at this concentration was shown to induce GPNT cell line toward a more differentiated phenotype (I. A. Romero et al., manuscript in preparation). At confluence, cells were washed with PBS and incubated for 48 h with or without increasing concentrations of the cytotoxic drug vincristine. After a 48-h incubation, 100 μl of a 1 mg/ml MTT solution was added to each well for 4 h. The medium was then removed, cells were washed with PBS, and the formazan precipitate was then dissolved by addition of 200 μl of 2-propanol. Absorbance was read at 540 nm using a Labsystems Multiskan RC micro plate reader. Cell viability was evaluated as EC_{50}, i.e., the drug

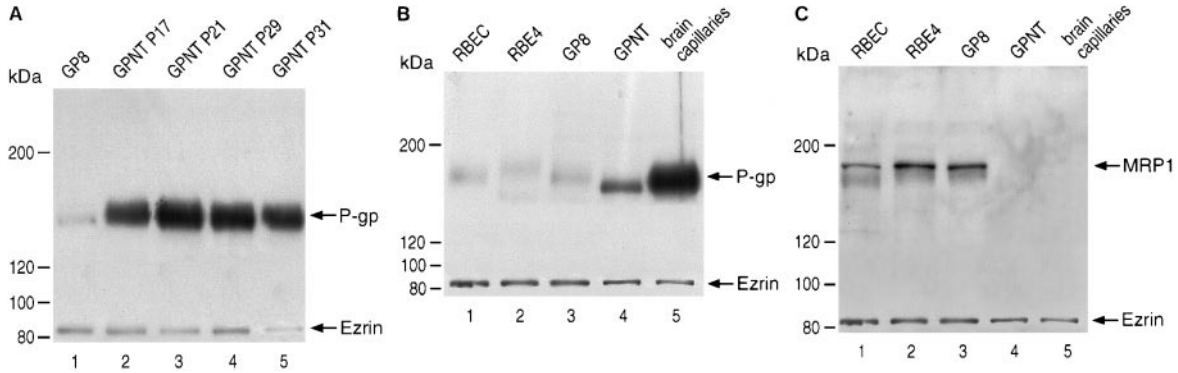

FIG. 1. Immunodetection of (**A** and **B**) P-gp and (**C**) Mrp1. Membranes were probed with (A and B) monoclonal anti-P-gp antibody C219 and (C) polyclonal anti-Mrp1 antiserum 6KQ. Protein samples (25 μg) were loaded on SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. A: GP8 cells (lane 1) and GPNT cells at passages 17, 21, 29, and 31 (lanes 2–5, respectively). B and C: Rat brain isolated capillaries (lane 5) and RBEC, RBE4, GP8, and GPNT cells (lanes 1–4, respectively). To verify equal protein loading, membranes were probed with an anti-ezrin antibody.

concentration providing a 50% reduction in absorbance as compared with control cells cultured without cytotoxic drug.

P-gp activity and expression. GPNT cells were incubated with dexamethasone (1 μM) for varying intervals (from 1 h to 3 days) and with dexamethasone at increasing concentrations (0.01–1 μM) for 72 h. Dexamethasone was stored as a 1 m*M* stock solution in ethanol at −80°C. Control cells were treated with 0.1% ethanol. Cells were washed with PBS and incubated in dexamethasone-free medium, and the uptake of vincristine was measured as described above. Immunoblots were performed in parallel as described above.

PKC modulation

Confluent GPNT cells were cultivated for 24 h with serum-free medium supplemented with 1% BSA. This 24-h culture did not change the basal vincristine accumulation in P-gp activity experiments.

PKC activity was stimulated by incubating GPNT cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 160 n*M*) (Stanimirovic et al., 1995) for short intervals (15–60 min of treatment). The effects of PKC inhibition on P-gp activity were evaluated by prolonged exposure to TPA (overnight treatment) or by incubation with the PKC inhibitor bisindolylmaleimide GF 109203X (0.1 μM; Calbiochem, France Biochem, Meudon, France) (Toullec et al., 1991). Vincristine accumulation was measured as described above.

Statistical analysis

One-way ANOVA followed by multiple comparisons using the Bonferroni adjustment were performed. In all cases, significance levels were set at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

Expression of P-gp and Mrp1 in GPNT cells

The immortalized brain endothelial cell line GP8 has been previously characterized: It displays a nontransformed phenotype highly reminiscent of BBB endothelium (Greenwood et al., 1996). To improve further the stability of this phenotype, GP8 cells were retransfected with a plasmid containing a puromycin resistance gene for selection. After limited dilution, a subclone was selected and called GPNT. Unlike the parental clone GP8, GPNT cells were cultured up to passage 50 without apparent morphological changes and displayed a highly stable phenotype (I. A. Romero et al., manuscript in preparation). We then investigated the level of expression of P-gp and Mrp1 by immunoblot analysis (Fig. 1).

In both GP8 and GPNT cells (Fig. 1A, lanes 1 and 2), P-gp was detected with a molecular size of 150–160 kDa, but the level of expression was much higher in GPNT cells compared with the parent GP8 cell line. The high level of P-gp expression in GPNT cells was maintained through passages 17–31 (Fig. 1A, lanes 2–5). The level of expression of P-gp was also much higher than in brain endothelial cells in primary culture or in RBE4 cells (Fig. 1B, lanes 1 and 2). However, the level of expression of P-gp in GPNT cells was still lower compared with the level observed in isolated capillaries (Fig. 1B, lane 5), further confirming the high level of P-gp expression in brain endothelium in vivo.

As previously reported (Regina et al., 1998), using the polyclonal antibody 6KQ, the expression of Mrp1 was detected in rat brain endothelial cells in primary culture and in RBE4 cells (Fig. 1C, lanes 1 and 2); Mrp1 expression was also detected in GP8 cells (Fig. 1C, lane 3) with a single immunoreactive band of ~170–180 kDa. However, at the same protein concentration (25 μg), no Mrp1 expression was detected in GPNT cells (Fig. 1C, lane 4) or in isolated capillaries (Fig. 1C, lane 5). In a previous report, 100 μg of protein was used to detect Mrp1 in isolated capillaries (Regina et al., 1998).

Functional analysis of P-gp activity

Vincristine and colchicine accumulation in GPNT cells. As a measure of drug efflux activity in GPNT cells, [^{3}H]vincristine and [^{3}H]colchicine accumulations were monitored following a 1-h exposure. The uptake of [^{3}H]vincristine into GPNT cells is shown in Fig. 2A. Vincristine accumulation was significantly increased following preincubation of GPNT cells with the P-gp inhibitors cyclosporin A (10 μM) and verapamil (10 μM)

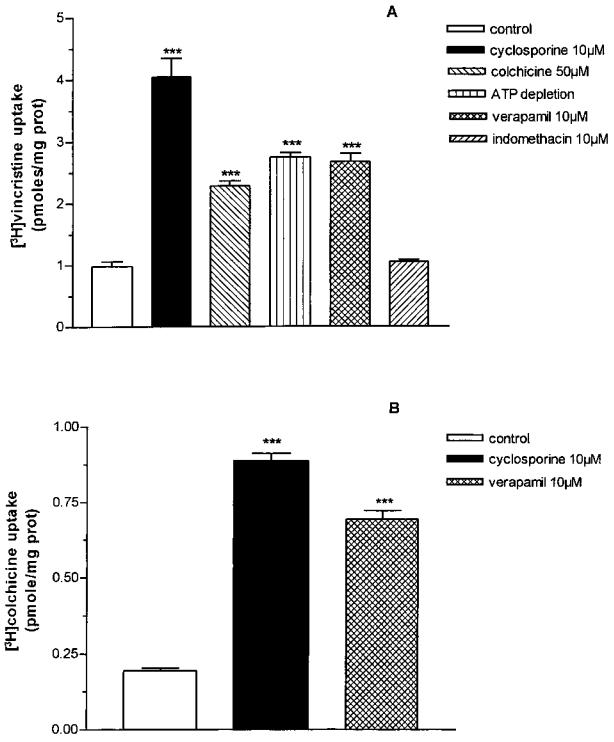

FIG. 2. Effects of P-gp- and MRP-specific modulators on (**A**) vincristine and (**B**) colchicine accumulation in GPNT cells. Cells were preincubated for 30 min at 37°C in growth medium alone (control) or growth medium containing 10 μM cyclosporin A (A and B), 50 μM colchicine (A), 10 μM verapamil (A and B), or 10 μM indomethacin (A). For ATP depletion (A), cells were preincubated in growth medium with 10 m*M* deoxyglucose and 10 m*M* sodium azide. Then 10 n*M* [^{3}H]vincristine or 10 n*M* [^{3}H]colchicine was added, and uptake was measured after a 1-h incubation. Data are mean ± SEM (bars) values, in pmol of drugs/mg of protein measured in the cells, of three independent experiments performed in quadruplicate.

(about four and 2.5 times, respectively; $p < 0.001$). By contrast, preincubation with the MRP inhibitor indomethacin (10 μM) had no effect on vincristine accumulation. Preincubation of GPNT cells with the P-gp substrate colchicine (50 μM) significantly increased vincristine accumulation (2.2 times; $p < 0.001$). Intracellular ATP depletion induced a large increase in vincristine accumulation in GPNT cells (2.5 times; $p < 0.001$). In addition, we investigated the accumulation of colchicine in GPNT cells (Fig. 2B). Colchicine accumulation was significantly increased by preincubation with 10 μM cyclosporin A and 10 μM verapamil (about eight and seven times, respectively; $p < 0.001$).

Permeability of GPNT monolayers. The passage of [^{3}H]vincristine and of the paracellular permeability tracer fluorescein was measured across monolayers of GPNT cells grown on collagen-fibronectin-coated Transwell-clear filters. A representative transport assay using triplicate filters is shown in Fig. 3. The tracer flux was measured over a 60-min period in the presence or absence of the P-gp blocker cyclosporin A, and the cleared volumes (in μl) were plotted against time.

The mean ± SEM P_e in GPNT cells for fluorescein was 7.08 ± 0.270 × 10^{-3} cm min^{-1} (n = 9 from three independent assays). Preincubation of cell monolayers with cyclosporin A had no effect on fluorescein permeability (6.99 ± 0.22 × 10^{-3} cm min^{-1}, n = 9 from three independent assays), indicating that cyclosporin A did not induce permeability changes in the endothelial monolayer (Fig. 3B). However, the apical-to-basal transport of vincristine was low despite its high lipophilicity (mean ± SEM $P_{e,vincristine}$ = 2.672 ± 0.03 × 10^{-3} cm min^{-1}, n = 9 from three independent assays). Preincubation of cell monolayers with the P-gp blocker cyclosporin A significantly increased the mean ± SEM $P_{e,vincristine}$ value to 10.2 ± 0.41 × 10^{-3} cm min^{-1} ($p < 0.01$, n = 9 from three independent assays) (Fig. 3A).

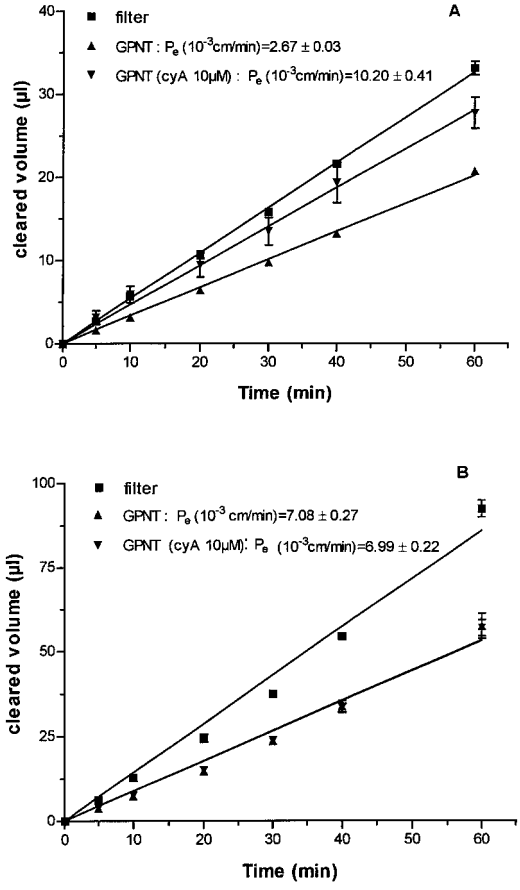

FIG. 3. Effect of cyclosporin A (cyA) on apical-to-basal transport of (**A**) vincristine and (**B**) fluorescein across GPNT monolayers. Confluent monolayers of GPNT cells grown on Transwell-clear filters were incubated with DMEM without phenol red containing 10 μM cyA for 30 min. Then, DMEM containing [^{3}H]vincristine (final concentration, 1 μCi/ml) and fluorescein (final concentration, 1 mg/ml) was added to the upper chamber. The fluxes of vincristine and fluorescein were measured by quantitating the amount of solute in the basal compartment. Cleared volumes of both tracers were plotted versus time for filters without cells (■), GPNT cells (▲), and GPNT cells preincubated with cyA (▼). Data are mean ± SEM (bars) values of triplicate filters of one representative experiment of three.

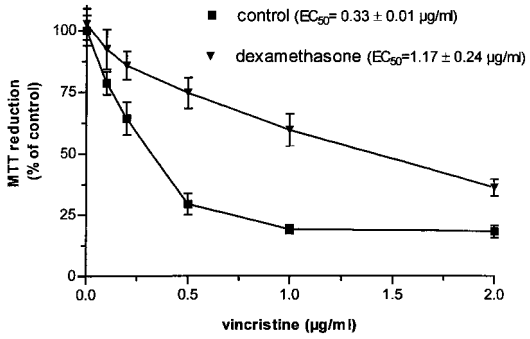

FIG. 4. Dexamethasone effect on vincristine cytotoxicity in GPNT cells. GPNT cells were plated in 96-well plates with or without dexamethasone (1 μM). At confluence, cells were washed with PBS and incubated for 48 h with increasing concentrations of vincristine. After incubation, 100 μl of a 1 mg/ml MTT solution was added to each well for 4 h, and MTT reduction was measured. Cell viability was evaluated as EC_{50}, i.e., the drug concentration providing a 50% reduction in absorbance as compared with control cells cultured without vincristine. Data are mean ± SEM (bars) values of three independent experiments.

At the end of the experiment, intracellular accumulation of both fluorescein and vincristine was always <0.1% of the initial amount of tracer placed on the apical side.

Modulation of P-gp activity by dexamethasone

Chemosensitivity assay. The effect of dexamethasone, a glucocorticoid previously shown to up-regulate P-gp expression in hepatoma cells (Zhao et al., 1993), on vincristine cytotoxicity was investigated in GPNT cells using the MTT cell viability assay (Fig. 4). GPNT cells pretreated with dexamethasone (10^{-6} M for 3 days) were significantly less susceptible to vincristine cytotoxicity with EC_{50} values of 1.17 ± 0.24 μg/ml compared with control values of 0.33 ± 0.01 μg/ml (n = 27 from three independent assays, $p < 0.05$). Thus, treatment with dexamethasone leads to an increase in resistance of GPNT cells to the cytotoxic effect of the P-gp substrate vincristine.

P-gp activity and expression. To investigate whether the protective effect of dexamethasone was the result of a modulation of P-gp function in GPNT cells, we measured the accumulation of vincristine after treatment with dexamethasone (1 μM) for various intervals or after a 72-h treatment with dexamethasone at various concentrations (1–0.01 μM). Dexamethasone concentrations used were in the range of concentrations used by Zhao et al. (1993) and Fardel et al. (1993). As shown in Fig. 5A, [^{3}H]vincristine accumulation was decreased in GPNT cells as early as 1 h following treatment with 1 μM dexamethasone. The decrease in vincristine uptake was even more pronounced after the cells had been exposed for longer intervals to dexamethasone, with a maximum at 72 h leading to a 50% decrease in vincristine uptake. A 50% decrease in vincristine accumulation was also observed after treatment with 0.1 μM dexamethasone; a 30% decrease was observed after treatment with 0.01 μM (data not shown). The increased drug efflux activity induced by dexamethasone was not due to an increase in P-gp expression by GPNT cells, as evidenced by semi-quantitative immunoblotting (Fig. 5B). Thus, treatment with dexamethasone induces an increase in drug efflux activity in the GPNT cell line, suggesting increased P-gp activity.

Role of PKC in dexamethasone-induced P-gp activation. It has been previously reported that PKC may activate P-gp activity in tumor cells (Aftab et al., 1994). Therefore, we assessed the role of PKC in dexamethasone-induced P-gp activity in GPNT cells. Activation of PKC with 160 nM TPA for 1 h decreased the accumulation of vincristine by 30% in GPNT cells, a decrease similar to that obtained after a 1-h treatment with dexamethasone (Fig. 6A). By contrast, down-regulation of PKC activity by prolonged treatment with 160 nM TPA completely abolished the dexamethasone-induced decrease in vincristine accumulation in GPNT cells (Fig. 6A). Similarly, pretreatment of GPNT cells with the PKC-specific inhibitor bisindolylmaleimide GF 109203X (0.1 μM) inhibited P-gp activation by dexamethasone (Fig. 6B). Altogether, these results suggest that dexamethasone-induced P-gp activation likely involves a PKC-dependent mechanism.

DISCUSSION

In this study, we investigated the levels of expression and activity of the two drug efflux pumps, P-gp and

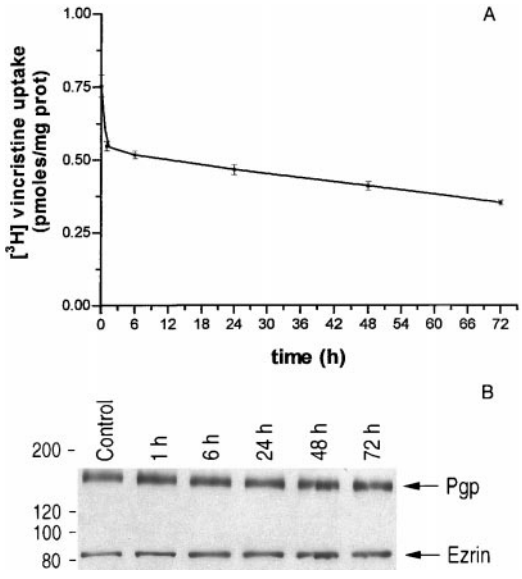

FIG. 5. A: Effect of dexamethasone treatment on vincristine accumulation in GPNT cells. Confluent GPNT cells were treated for increasing intervals with dexamethasone (10^{-6} M), and accumulation of [^{3}H]vincristine was measured. **B:** Effect of dexamethasone treatment on P-gp expression in GPNT cells. Protein samples of control cells and dexamethasone-treated cells were obtained as described. Protein samples (25 μg) were loaded on SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane for successive blotting with anti-P-gp C219 and anti-ezrin antibody.

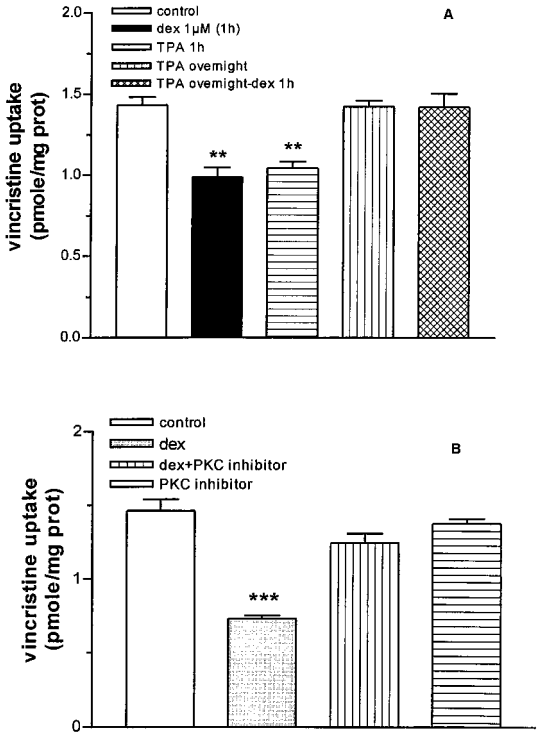

FIG. 6. Effect of PKC modulation on vincristine accumulation in GPNT cells. Confluent cells were cultivated for 24 h with serum-free medium supplemented with 1% BSA. **A:** GPNT cells were preincubated for 1 h with TPA (160 n*M*) or dexamethasone (dex) or with TPA (160 n*M*) overnight with or without a further 1-h dex incubation. **B:** Cells were preincubated with the PKC inhibitor bisindolylmaleimide GF 109203X (0.1 μM) for 1 h with or without a further 1-h incubation with dex. Cells were washed with PBS, medium was changed to medium without modulators, 10 n*M* [^{3}H]vincristine was added, and the uptake was measured after a 1-h incubation. Data are mean ± SEM (bars) values, in pmol of vincristine/mg of protein measured in the cells, of one representative experiment performed in quadruplicate.

Mrp1, in the immortalized rat brain endothelial cell line GPNT. We demonstrated that P-gp was highly expressed and active in these cells, whereas no expression or activity of Mrp1 was found. We analyzed the regulation of P-gp activity by the glucocorticoid dexamethasone, and we showed that in the GPNT model, dexamethasone increased P-gp activity, without inducing any increase in protein expression, but probably by a mechanism involving PKC activity.

It has been reported that culture conditions induce down-regulation of P-gp expression in brain endothelial cells (Barrand et al., 1995; Regina et al., 1998). In a previously described BBB in vitro model using bovine brain endothelial cells in primary culture, coculture with astrocytes was necessary to induce an increased expression of P-gp (Fénart et al., 1998). It is interesting that P-gp expression was much higher in GPNT cells than in the parent GP8 cell line and that this high expression was maintained through >45 doublings (15 passages) in solo cultures, at a level close to the in vivo situation.

This high expression correlated also with a high P-gp activity, as shown by drug accumulation as well as drug permeability studies. Agents known to inhibit P-gp drug efflux activity, cyclosporin A and verapamil, induced significant increase in intracellular accumulation of the cytotoxic P-gp substrates vincristine and colchicine; this increase was severalfold higher than that previously reported with human or rat brain endothelial cells in primary culture (Regina et al., 1998; Seetharaman et al., 1998). In addition, apical-to-basal transcellular transport of vincristine across GPNT monolayers was found to be extremely low, confirming the high efficiency of P-gp in these cells. Indeed, the permeability coefficient of GPNT cells for vincristine ($P_e = 2.67 \times 10^{-3}$ cm min^{-1}) is close to the permeability coefficient of the in vitro BBB model mentioned above, consisting of bovine brain endothelial cells cocultured with astrocytes ($P_e = 2.41 \times 10^{-3}$ cm min^{-1}) (Fénart et al., 1998).

GPNT cells did not appear to contain detectable levels of the multidrug transporter Mrp1. In addition, the accumulation of vincristine, a known substrate of both transporters P-gp and MRP, was not affected by the specific MRP inhibitor indomethacin, confirming that GPNT cells did not express MRP activity. Although Mrp1 has been found to be expressed and functional in a mouse brain capillary endothelial cell line (Kusuhara et al., 1998) and in primary bovine brain endothelial cells (Huai-Yun et al., 1998), this activity likely reflects artifactual overexpression of the Mrp1 protein due to in vitro culture conditions. Likewise, in human brain endothelial cells, MRP1 expression has not been observed in freshly isolated capillaries, whereas MRP expression/activity could be demonstrated in primary cultured cells grown from those capillaries (Seetharaman et al., 1998). In a previous report, we also observed a weaker Mrp1 expression in freshly isolated rat brain capillaries than in the corresponding cultured endothelial cells derived from these capillaries (Regina et al., 1998). This weak Mrp1 expression in isolated capillaries could be demonstrated only when 100 μg of protein was loaded on the gel. Furthermore, Mrp1 expression was reduced, and MRP activity was not observed anymore in these endothelial cells cocultured with astrocytes, corresponding to enhanced differentiation of cultured brain endothelial cells (Regina et al., 1998). Thus, high expression/activity of P-gp and absence of Mrp1 expression/activity would indicate that the GPNT cell line may be a useful model for analyzing P-gp properties at the BBB.

The ability of dexamethasone to modulate physiological P-gp activity is of clinical interest. Indeed, glucocorticoids are usually included in chemotherapy regimens for the treatment of brain tumors, because of their capacity to reduce brain tumor-associated vascular permeability and cerebral edema. Tumor-associated cerebral edema is a consequence of vascular endothelial growth factor production by brain tumor cells. Indeed, vascular endothelial growth factor acts on endothelial cells by inducing neoangiogenesis and by increasing vascular permeability (Roberts and Palade, 1995). One mecha-

nism by which dexamethasone reduces brain edema involves the reduction of permeability factor expression by tumor cells. Indeed, dexamethasone has already been shown to reduce vascular endothelial growth factor expression by tumor cells (Heiss et al., 1996; MacHein et al., 1999). Another possible mechanism for dexamethasone to reduce brain edema would be a direct effect on endothelial cells. Previous results using the GPNT cell line have indicated that treatment with dexamethasone may induce further differentiation of this cell line toward a BBB phenotype (I. A. Romero et al., manuscript in preparation). Indeed, the permeability to low-molecular-weight tracers was decreased, and the pattern of expression of the tight junction protein ZO-1 appeared continuous after dexamethasone treatment. In the present study, dexamethasone treatment increased GPNT resistance to the cytotoxic drug vincristine, together with higher P-gp activity. However, such an increase in P-gp activity was not associated with up-regulation of P-gp expression, suggesting posttranslational regulation in agreement with previous observations in primary cultures of rat hepatocytes (Chieli et al., 1994). These results suggest an induction of P-gp activity and BBB characteristics in dexamethasone-treated brain endothelial cells must be confirmed in vivo. Indeed, such a dual effect of dexamethasone treatment on brain endothelial cells would be prejudicial for patients with malignant brain tumors with regard to systemic chemotherapy regimens.

Regulatory mechanisms of P-gp activity have not been fully elucidated, but one potential candidate is the phosphorylation of this protein. Several studies in vitro have shown that P-gp is phosphorylated in the linker region, between the two domains of the molecule, by several serine–threonine kinases, including PKC (Meyers, 1989; Chambers et al., 1990, 1993; Aftab et al., 1994; Glavy et al., 1997). However, whether PKC-dependent phosphorylation of P-gp modulates its drug efflux activity remains controversial. Although in some studies P-gp activity has been shown to be preserved when the PKC phosphorylation sites of the linker region are mutated (Goodfellow et al., 1996), in other studies phosphorylation of P-gp has been associated with a significant increase in its drug efflux activity (Chambers et al., 1992; Matsumoto et al., 1995; Ramachandran et al., 1998). According to Szabó et al. (1997), phosphorylation of the linker region could modulate the interaction of drugs with P-gp, especially at low concentrations, and might therefore regulate the P-gp drug efflux activity without altering the maximal level of P-gp-ATPase activity. Some studies have demonstrated a dexamethasone-induced translocation and activation of PKC in different cell types, such as adipocytes and soleus muscle (Ishizuka et al., 1995), aortic smooth cells (Kato et al., 1992), and renal epithelial cells (Vrtovsnik et al., 1994). In our study, activation of PKC by TPA increased P-gp activity. By contrast, PKC down-regulation by prolonged TPA treatment, as well as direct inhibition by bisindolylmaleimide, abolished the dexamethasone-induced increase of P-gp activity. Taken together, these results suggest that, in rat brain endothelial cells, dexamethasone increases P-gp drug efflux activity by increasing PKC-mediated phosphorylation of P-gp.

In conclusion, we have shown that GPNT cells retain a P-gp and Mrp1 expression pattern close to that of BBB endothelial cells in vivo. These cells express a highly active P-gp, which can be further activated by glucocorticoid treatment, possibly through PKC-dependent P-gp phosphorylation. Taking into account the advantages of a stable immortalized cell line in solo culture over a co-culture of primary cells, in terms of large-scale production and standardization, the GPNT cell line model can be considered as a valuable in vitro model to investigate the passage of P-gp-dependent drugs into brain parenchyma or the modes of regulation of P-gp activity.

Acknowledgment: This research was supported by grants MLD/CM/ML-CN3/98 from the Association pour la Recherche sur le Cancer and a grant from the European Commission (Biomedicine Program).

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