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Role of Protein Kinase C in the Modulation of Multidrug Resistance: Expression of the Atypical γ Isoform of Protein Kinase C Does not Confer Increased Resistance to Doxorubicin

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SUMMARY

Cross-resistance to anticancer drugs, termed multidrug resistance (MDR), is functionally associated with the expression of a plasma membrane, energy-dependent, drug efflux pump termed P-glycoprotein (PGP), the product of the *mdr1* gene. We have shown previously that MCF-7 breast carcinoma cells transfected with the human *mdr1* gene (BC-19 cells) exhibit greater MDR when stably transfected with protein kinase Cα (PKCα). We now demonstrate that transfection of BC-19 cells with the γ isoform of PKC (BC-19/PKCγ cells), which is not normally present in BC-19 cells, does not confer increased resistance to doxorubicin, despite a 19-fold increase in PKC activity. All of the increased PKC activity is accounted for by PKCγ and it is rapidly down-regulated by phorbol dibutyrate, within 15 min of treatment. Endogenous PKCα and PKCε activities are not affected by phorbol dibutyrate. The cytotoxicity of doxorubicin was similar in BC-19/*neo* or BC-19/PKCγ cells after either 2-hr or continuous drug exposure, and co-treatment with phorbol dibutyrate increased resistance to doxorubicin 4-fold in both cell lines. Phosphorylation of PGP was similar in both cell lines and drug accumulation was not affected by overexpression of PKCγ. These results demonstrate that transfection of PGP-expressing cells with an atypical isoform of PKC does not confer increased MDR, and they suggest that the regulation of PGP is phenotype specific with respect to the isoform of PKC.

MDR is associated with the elevated expression of the plasma membrane protein PGP, the product of the *mdr1* gene (1–4). There is considerable genetic evidence that a wide variety of animal and human MDR cells exhibit amplification and overexpression of the *mdr1* gene (5–9). Transfection studies with both genomic and cDNA copies of the *mdr1* gene have confirmed that it confers the MDR phenotype to the recipient cells (10–15), although the degree of resistance is considerably less than that in naturally selected resistant cell lines. The involvement of PGP in drug resistance is believed to reside in its ability to function as an ATP-dependent drug efflux pump. This conclusion is based on the ability of a monoclonal antibody to PGP, MRK16, to partially restore drug sensitivity through enhancement of net drug uptake (16), on protein sequence homology to a bacterial active transport protein (17–19), and on the demonstration of PGP-associated ATPase activity (20), unidirectional drug efflux (21), and ATP-dependent drug binding (22).

Although there is overwhelming evidence that PGP is a major factor in MDR, the role of post-translational modification of this protein in the resistance process is less certain. One of the earliest metabolic changes noted in PGP was its phosphorylation in colchicine- or doxorubicin-resistant cells but not in the parental or revertant cell lines (23–25). Other investigators have noted this characteristic in a variety of rodent and human MDR cell lines (26–29). The protein kinase responsible for the phosphorylation of PGP has not been identified, although the PKC activator PDBu stimulates the phosphorylation of PGP *in vivo* (26), and most cell lines selected for MDR contain increased levels of PKC (30–37). Perhaps the best evidence for the involvement of PKC in MDR is the recent study showing that transfection of BC-19 cells with the cDNA for PKCα conferred increased resistance to doxorubicin and vinblastine, decreased drug retention, and increased phosphorylation of PGP (38). Because BC-19 cells were derived from MCF-7 cells by transfection with the human *mdr1* cDNA (39) and contained

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ABBREVIATIONS: MDR, multidrug-resistant (or resistance); PGP, P-glycoprotein; PKC, protein kinase C; PDBu, phorbol dibutyrate; RSV, Rous sarcoma virus; PMSF, phenylmethylsulfonyl fluoride; Kb, kilobase(s); PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

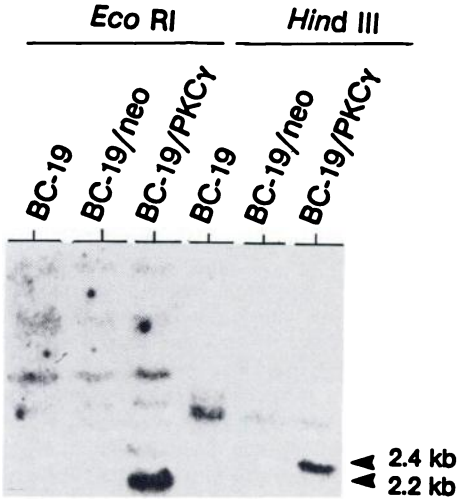


Fig. 1. Expression of the PKCγ cDNA in transfected BC-19 cells. G418-resistant BC-19 cells were isolated after transfection with pRSVneo or pRSV-PKCγ and were analyzed by Southern blotting. Each lane contained 5 μg of DNA digested with either *Eco*RI or *Hind*III and hybridization was carried out with the *Hind*III PKCγ cDNA as a probe. *Upper arrowhead*, the 2.4-kb PKCγ cDNA resulting from digestion with *Hind*III; *lower arrowhead*, the 2.2-kb PKCγ cDNA fragment resulting from digestion with *Eco*RI.

very low levels of PKCα (39), this study was the first to demonstrate that overexpression of an endogenous isoform of PKC could confer increased MDR by modulation of PGP.

In the present investigation, the role of protein phosphorylation in MDR was examined in BC-19 cells transfected with a plasmid containing the cDNA for PKCγ, an isoform that is not normally present in this cell line. The results of this study demonstrate that overexpression of an atypical isoform of PKC is unable to modulate PGP activity and resistance to doxorubicin.

Experimental Procedures

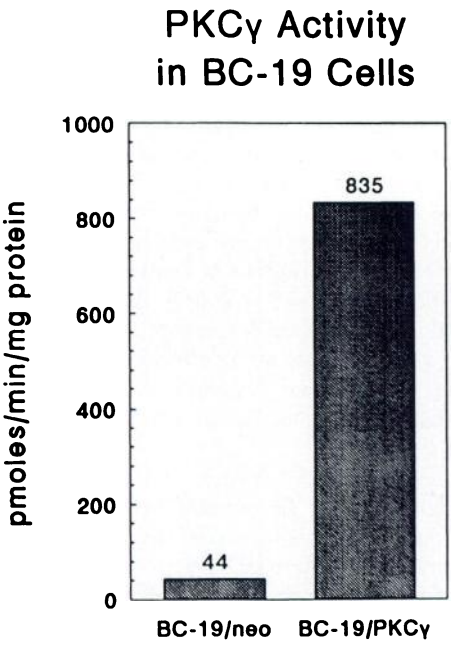
Cell lines. Cell line BC-19 was obtained from MCF-7 cells transfected with the *mdr1* gene and was selected on the basis of its resistance to doxorubicin (39). BC-19 cells were further subcloned and an isolate exhibiting a uniform content of PGP was used throughout the present investigation. The doubling time for either BC-19 cells or the various transfectants was approximately 24 hr, similar to that of MCF-7 cells.

Cytotoxicity assays. Cytotoxicity was determined by clonogenic assay after either 2 hr or continuous exposure to doxorubicin (38). Cells were plated in four-well Linbro plates at a density of 200 cells/well in Iscove's medium with 10% heat-inactivated fetal bovine serum 12 hr before the experiments, to allow cells to adhere firmly to the surface of the plates. After removal of the medium, the cells were re-fed with fresh Iscove's medium containing varying concentrations of doxorubicin with or without 200 nM PDBu. In experiments in which cells were exposed to doxorubicin for 2 hr, the drug-containing medium was removed after 2 hr and cells were re-fed with drug-free medium. After about 2 weeks, colonies were fixed in methanol, stained with Giemsa stain, and counted visually.

Transfection of BC-19 cells with pRSV-PKCγ. The cDNA for PKCγ (40) was cloned into the *Hind*III site of plasmid pRSVneo (41) 3′ to the RSV promoter and 5′ to the polyadenylation sequence. Lipofectin (Life Technologies, Inc.) was used for transfection according to the instructions from the manufacturer, except that the ratio of DNA to Lipofectin was maintained at 1:2 and 25 μg of plasmid and 50 μg of lipofectin were used for each transfection, in 60-mm Falcon dishes. Stable transfectants were initially selected in 1 mg/ml G418 (Life Technologies, Inc.), which was subsequently increased to 1.5 mg/ml in the second through fourth week of selection. Cells surviving selection were expanded in the absence of G418 for the present studies.

PKC assay. Logarithmic phase cells ($1–2 \times 10^7$) were harvested 3 days after seeding and cell extracts were prepared as described previously (38). The supernatant was adsorbed to 0.25 ml of DEAE-Sepharose Fast Flow equilibrated in buffer A (20 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM EGTA, 0.5 mM EDTA) in a 1.5-ml microcentrifuge tube, washed three times with buffer A, and eluted with 0.2 M NaCl in buffer A. PKC was assayed as described previously (38), except that the PKC-specific peptide [Ser^{25}]PKC_{19-31} (Life Technologies, Inc.) was used in place of histone H1.

Immunoblot for PKCγ. DEAE-Sepharose-eluted protein (50 μg) was separated by SDS-PAGE in 10% polyacrylamide gels (Novex Inc.) and electroblotted onto nitrocellulose as described previously (38). Blots were incubated with affinity-purified rabbit polyclonal antibodies against the α, β, γ, δ, ε, and ζ isoforms of PKC (Life Technologies, Inc.) and immunoreactive protein was visualized with goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad), using nitroblue



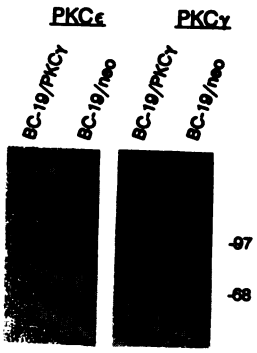


Fig. 2. PKC activity and PKCγ and PKCε levels in transfected BC-19 cells. *Left*, activity was measured in total cellular extracts after DEAE-Sepharose extraction, as described in Experimental Procedures. Each PKC activity value is the mean of three experiments. *Right*, cell extracts were separated by SDS-PAGE in a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with a rabbit polyclonal antibody against either PKCε or PKCγ as the primary antibody. *Numbers on the right*, molecular mass standards (in kDa). Western blots contained 50 μg of protein/lane.

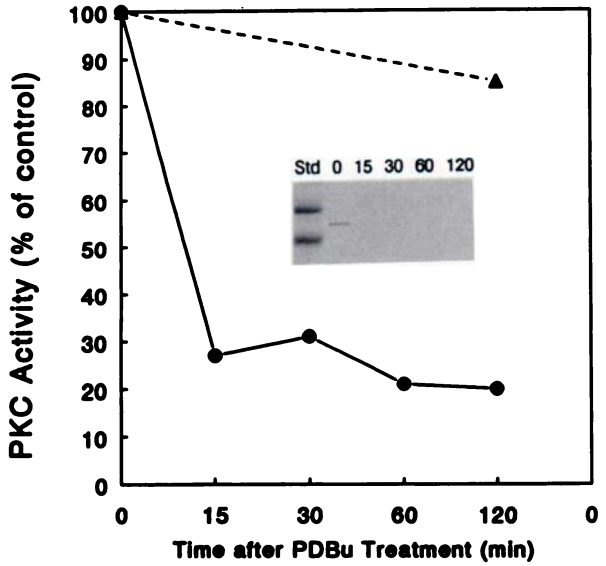


Fig. 3. Effect of PDBu on the level of PKCγ and PKC activity. Immunoblotting (*inset*) and PKC activity were determined as described in Experimental Procedures.

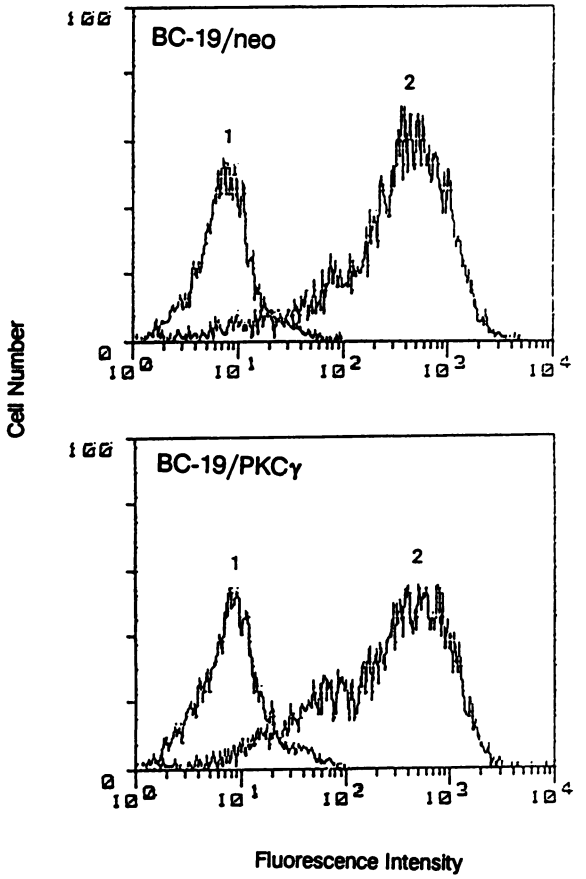


Fig. 4. Flow cytometry analysis of PGP in transfected BC-19 cells. Flow cytometry was performed with monoclonal antibody MRK16, as described in Experimental Procedures. *1*, Cells incubated with fluorescein-conjugated goat anti-mouse IgG alone; *2*, cells incubated with MRK16 and fluorescein-conjugated goat anti-mouse IgG.

tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.) as the substrates.

PGP detection. PGP was quantitated by two procedures. Flow cytometry utilized monoclonal antibody MRK16. Logarithmic phase cells were removed from 25-cm² plastic flasks by treatment with 1 mM EDTA in PBS, pH 7.2. Cells were suspended (5×10^5/50 μl) in 50 μl of PBA (1% bovine serum albumin, 0.1% NaN_3, in PBS), incubated with 1 μg of MRK16 for 30 min at 4°, washed with PBA, incubated with fluorescein-conjugated goat anti-mouse immunoglobulin for 20 min at 4°, washed with PBA, and resuspended in PBA for immediate analysis. Negative controls were performed with each cell line, using fluorescein-conjugated goat anti-mouse IgG without MRK16. Analysis was performed on a FACStar flow cytometer interfaced with a Consort 30 data acquisition and analysis system (42).

The second procedure utilized an ELISA in which cells were plated into 96-well plates that had been precoated with poly-L-lysine and were incubated overnight to allow attachment of cells. Cells were washed once with PBS and incubated for 1 hr with MRK16 at a concentration of 2.5 μg/well. Cells were washed three times with PBS and incubated for 1 hr with goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad) (1/2000 dilution), and the reaction was developed with a TMB peroxidase substrate kit (Bio-Rad) according to the manufacturer's instructions.

Drug retention. The cellular uptake of [³H]vinblastine was measured as described previously (32).

***In vivo* phosphorylation of PGP.** Cells were incubated in 10 ml of phosphate-free Iscove's modified Dulbecco's minimal essential medium containing 40 mM HEPES, pH 7.4, and 50 μg/ml gentamicin in 175-cm² plastic flasks for 30 min before labeling for 4 hr with 0.25 mCi/ml $H_3{}^{32}PO_4$. PDBu (200 nM) or an equivalent amount of dimethylsulfoxide (final concentration, 0.1%) was added during the last 2 hr of incubation. Cells were harvested by scraping, washed in ice-cold Hanks' balanced salt solution without Mg^{2+} or Ca^{2+} and with 20 mM EDTA, homogenized in buffer containing 10 mM Tris·HCl, pH 7.5, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 2 mM sodium vanadate, 10 mM NaF, and 1 mM ATP, and centrifuged at 5000 × *g* for 10 min. The supernatant was centrifuged at 100,000 × *g* for 1 hr, the pellet was dissolved in buffer S (50 mM Tris·HCl, pH 8.0, 140 mM NaCl, 5 mM NaF, 2 mM sodium vanadate, 0.2 mM PMSF, 0.2 mg/ml aprotinin, 4 mM EDTA, 0.5% sodium deoxycholate), and PGP was immunoprecipitated with monoclonal antibody MRK16 (26).

Results

PKC expression. BC-19 cells were subcloned from the original isolate to obtain a cell population that more uniformly expressed PGP. Cells were transfected either with an expression vector containing the RSV promoter and the *neo* resistance gene or with the same plasmid containing the cDNA for rabbit brain PKCγ (41). Transfected cells were selected for their resistance to G418 and examined for integration of the PKCγ cDNA by Southern blotting (Fig. 1). Restriction digestion of DNA from BC-19 cells expressing the *neo* locus of resistance (BC-19/*neo*) or BC-19 cells containing the *neo* and PKCγ genes (BC-19/PKCγ) with *Eco*RI indicated that only BC-19/PKCγ cells contained the 2.2-kb PKCγ cDNA fragment. Restriction with *Hind*III revealed a 2.4-kb fragment containing the PKCγ cDNA. Expression of the genomic sequence of PKCγ was similar in both BC-19 cell lines as well as in wild-type MCF-7 cells (Fig. 1).

BC-19/PKCγ cells expressed 19-fold greater PKC activity than did BC-19/*neo* cells (Fig. 2). Immunoblotting of cell extracts prepared from both cell lines indicated that PKCγ was the predominant isoform expressed in BC-19/PKCγ cells. The only other isoforms of PKC that were detectable in either cell line were PKCα (data not shown) and PKCε. The level of

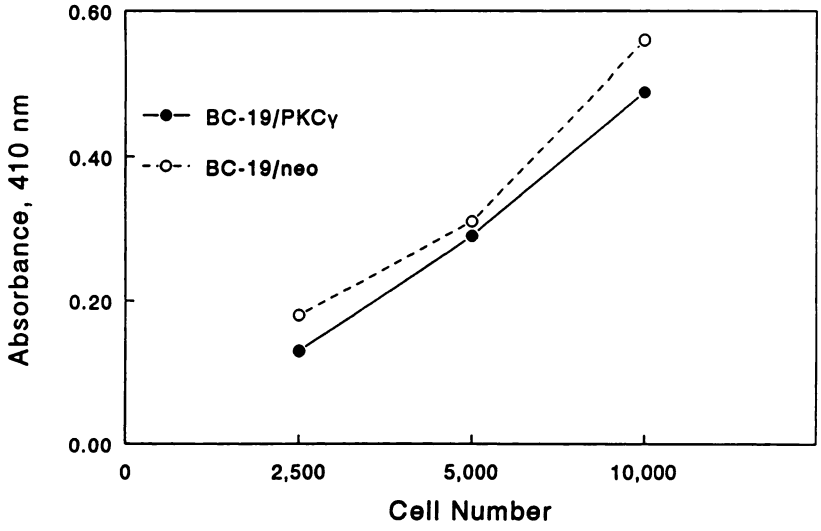


Fig. 5. ELISA for PGP in transfected BC-19 cells. Monolayers of BC-19/*neo* and BC-19/PKCγ were assessed for PGP levels with MRK16 as the primary antibody.

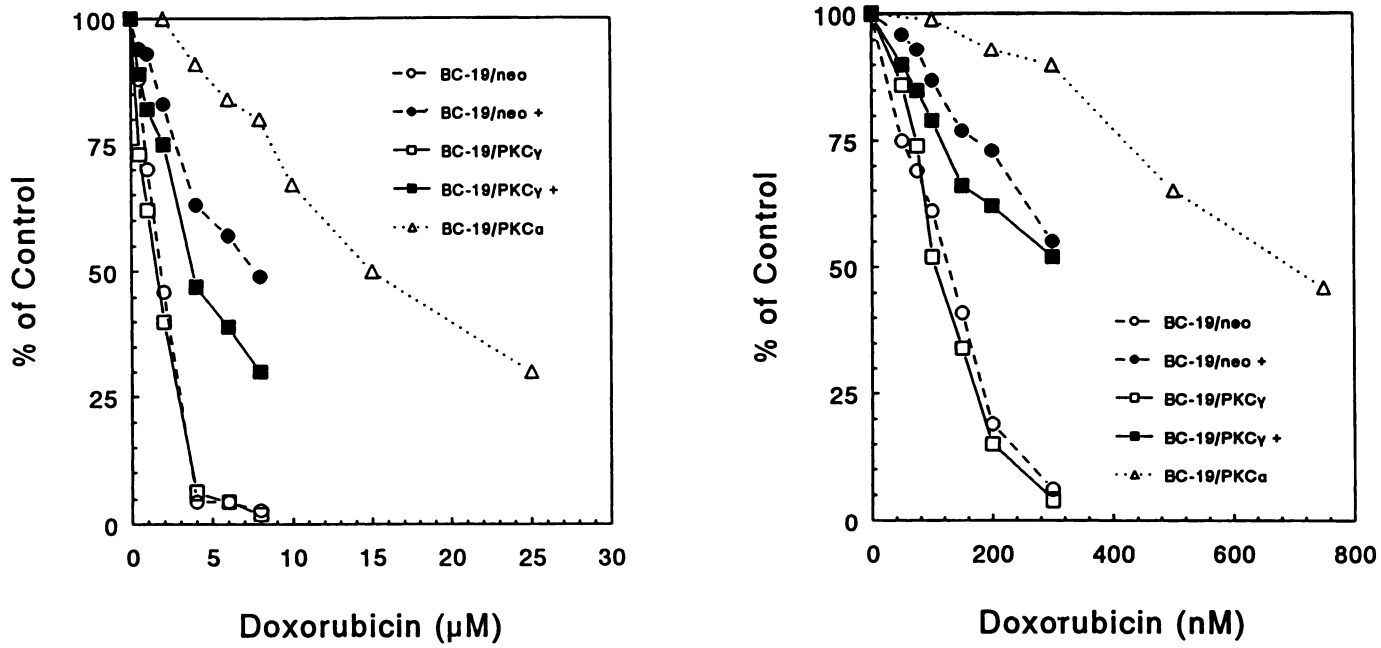


Fig. 6. Cytotoxicity of doxorubicin and PDBu in transfected BC-19 cells. BC-19/*neo* or BC-19/PKCγ cells were either exposed to doxorubicin or doxorubicin plus 200 nM PDBu (+) for 2 hr, washed, and re-fed with fresh drug-free medium (*left*) or exposed continuously for 10 days (*right*). Colony formation was determined after 10 days.

PKCα was barely discernable in either cell line (38) and BC-19/PKCγ cells contained somewhat lower levels of PKCε than did BC-19/*neo* cells. Unfortunately, the activity of PKCε could not be confirmed enzymatically, because no isoform-specific substrate is available.

Because PKCγ is known to be the isoform most susceptible to proteolysis among the α, β, and γ species (43, 44), PKC activity was measured after treatment of BC-19/*neo* and BC-19/PKCγ cells with PDBu (Fig. 3). PKC activity rapidly declined in BC-19/PKCγ cells as early as 15 min after exposure to PDBu, and the residual activity remaining after 2 hr represented approximately 20% of the total activity. In contrast, BC-19/*neo* cells lost only 15% of their PKC activity after 2 hr. Immunoblotting demonstrated that PKCγ completely disappeared by 15 min after treatment with PDBu (Fig. 3, *inset*). These results and those from immunoblotting suggest that the major isoform contained in the residual PKC after PDBu treatment is PKCε.

PGP levels. BC-19/*neo* and BC-19/PKCγ cells were analyzed for PGP levels by flow cytometry (Fig. 4) and by a more quantitative ELISA (Fig. 5). The representative cell population and median fluorescence were equivalent in both cell lines (Fig. 4). ELISAs also confirmed this result and showed identical levels of PGP (Fig. 5).

Drug sensitivity. BC-19/*neo* and BC-19/PKCγ cells were tested by colonogenic assay for their sensitivity to doxorubicin after either a 2-hr or a continuous exposure to drug (Fig. 6). After exposure of BC-19/*neo* and BC-19/PKCγ cells to doxorubicin for 2 hr, the IC_{50} was 1.9 and 1.6 μM doxorubicin, respectively. After exposure to PDBu, the IC_{50} was increased to 8 and 4 μM, respectively. A similar trend was noted after continuous exposure to doxorubicin, where the IC_{50} was 130 and 110 nM doxorubicin for BC-19/*neo* and BC-19/PKCγ cells, respectively, and 335 nM for either cell line after PDBu treatment. In contrast, BC-19 cells transfected with PKCα were

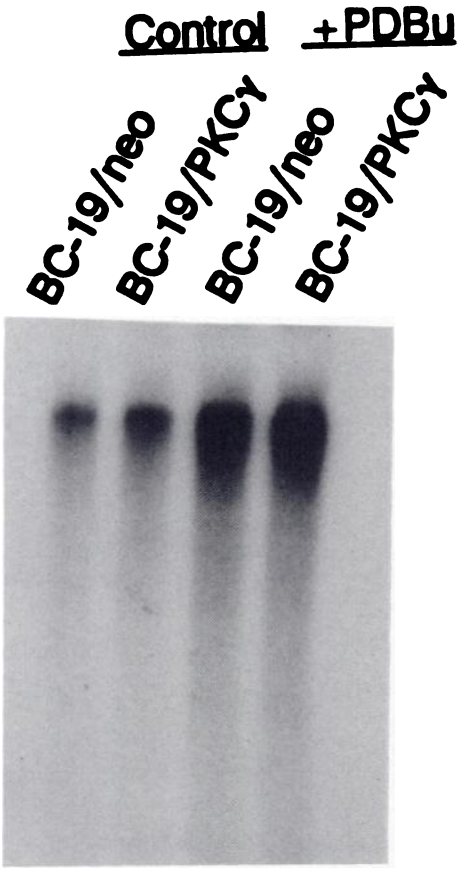


Fig. 7. Phosphorylation of PGP in transfected BC-19 cells. Cells were labeled with $H_3{}^{32}PO_4$ in the presence or absence of 200 nM PDBu, and immunoprecipitates were prepared with monoclonal antibody MRK16. Samples were separated by SDS-PAGE in an 8% polyacrylamide gel and were subjected to autoradiography.

approximately 7–8-fold more resistant to doxorubicin than were BC-19/*neo* cells.

Drug retention. The two cell lines were compared for their ability to accumulate doxorubicin. After exposure to [^{14}C]doxorubicin for 2 hr drug retention was not significantly different between BC-19/*neo* and BC-19/PKCγ cells, and simultaneous exposure to 200 nM PDBu did not decrease drug retention in either cell line (data not shown).

PGP phosphorylation. The phosphorylation of PGP was measured in BC-19/*neo* and BC-19/PKCγ cells by metabolic labeling with $H_3{}^{32}PO_4$ followed by immunoprecipitation of the membrane fraction, obtained from equal numbers of cells, with monoclonal antibody MRK16 (16) (Fig. 7). Both cell lines contained similar levels of phosphorylated PGP and phosphorylation was increased 3-fold after treatment for 2 hr with 200 nM PDBu.

Discussion

The involvement of PKC in the MDR phenotype has been implied by previous studies that demonstrated that many MDR cell lines contain higher PKC activity than do wild-type cells (30–37) and by the use of protein kinase inhibitors to decrease drug resistance (28, 36). Phorbol ester activators of PKC increased drug resistance in MCF-7 (32), KB (45), and chronic lymphocytic leukemia (46) cells, and their effect was associated with increased drug retention as well as activation and translocation of PKC (32). Phorbol esters also increased phosphorylation of PGP in doxorubicin-resistant (26) and vincristine-resistant (47, 48) cells, as well as in MDR BC-19 cells and BC-19 cells transfected with the PKCα cDNA (38). Because PGP is a substrate for PKC *in vitro* (46), these data suggest that PKC is involved in modulating PGP activity and the MDR phenotype.

The present study was designed to examine the effect of the atypical γ isoform of PKC on MDR in MCF-7 cells transfected with the *mdr1* gene (BC-19 cells). This required that the *neo* and PKCγ transfectants not only be homogeneous cell populations but also contain equivalent levels of PGP so that a comparison of cytotoxicity could be attributed to the overexpression of PKCγ and not PGP. The approach utilized a single clone of BC-19 cells that had a PGP content similar to that of the drug-selected MCF-7/ADR cell line (38, 39), and this was confirmed by flow cytometry analysis. The mass transfectants were then selected for 4 weeks in high concentrations of G418 to minimize effects due to clonal variation. The results of these experiments show that the atypical γ isoform of PKC in cells containing high levels of PGP does not lead to an increase in the level of resistance to doxorubicin. Regardless of whether cells were treated with PDBu, the cytotoxicity was similar between BC-19 cells transfected with a plasmid containing only the *neo* gene or with the same plasmid containing the cDNA for PKCγ. Although PDBu treatment did increase resistance 4-fold, the effect was similar in both cell lines regardless of the duration of treatment with doxorubicin. The lack of effect of overexpression of PKCγ in BC-19 cells may be explained by the rapid loss of PKCγ activity after PDBu treatment and demonstrates for the first time that the high sensitivity of PKCγ to proteolysis observed *in vitro* (43, 44) also occurs in intact cells. This characteristic of PKCγ, in contrast to the relative stability of PKCα to proteases (43, 44), suggests that the localization of this isoform predominantly in the central nervous system (49–52) is related to a signaling process that requires rapid abrogation of PKCγ activity.

Because drug retention, chemosensitivity, and PGP phosphorylation were unaffected by the overexpression of PKCγ, the present study also suggests that these hallmarks of the MDR phenotype are also dependent on the isoform of PKC expressed in the resistant cells. In this regard, it is interesting to note that overexpression of PKCγ resulted in reduced levels of PKCε (Fig. 2). Apparently, this is the first example of one isoform of PKC affecting the expression of another isoform of PKC. However, the significance of this effect to the MDR phenotype is not evident, because resistance was unaffected in BC-19/PKCγ cells. In contrast, HL-60/ADR cells, which do not contain PGP, expressed PKCγ, which is not normally expressed in the parental cell line (31) but may be induced by myeloid-differentiating agents (53). Therefore, the present study further demonstrates that the modulating effect of PKC on MDR is highly dependent on the isoform of PKC representative of the phenotype of the MDR cell line and is not simply a nonspecific phosphorylation process.

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