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Regulation of the Function of P-Glycoprotein by Epidermal Growth Factor through Phospholipase C

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ABSTRACT. Many multidrug-resistant (MDR) cell lines overexpress the epidermal growth factor receptor (EGFR) as well as P-glycoprotein (P-gp). However, the role of the increased EGFR in P-gp-mediated drug resistance remains unclear. Since recent studies suggest that activation of phospholipase C (PLC) could increase the phosphorylation of P-gp, and activation of the EGFR would also activate PLC, we investigated whether the effect of epidermal growth factor (EGF) on the phosphorylation of P-gp was mediated through PLC. Treatment of the human MDR breast cancer cell line, MCF-7/AdrR, with EGF increased the phosphorylation of P-gp by 20–50%. The increased phosphorylation of P-gp was accompanied by stimulation of PLC activity, as measured by the production of inositol, 1,4,5-trisphosphate and diacylglycerol, products of phosphatidylinositol-4,5-bisphosphate hydrolysis. Treatment of MDR cells with EGF also had detectable effects on P-gp function. For example, following incubation of MCF-7/AdrR cells with EGF, we observed a consistent decrease in total vinblastine (VBL) accumulation. Kinetic analysis revealed this change to be due to an increase in membrane efflux. The latter was measured by the initial uptake velocity, which was inhibited by EGF. VBL uptake measured at 0–320 sec was inhibited by 20–40%, which was associated with a similar increase in VBL efflux. EGF had no effect on drug accumulation, uptake, or efflux in sensitive MCF-7 cells. These data indicate that EGF can modulate the phosphorylation and function of P-gp, and suggest that this effect may be initiated by the activation of PLC. BIOCHEM PHARMACOL 53;11:1597–1604, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. P-glycoprotein; epidermal growth factor receptor; phospholipase C; signal transduction; breast cancer

Resistance to cytotoxic drugs is a major obstacle to successful cancer chemotherapy. One common form of MDR† is mediated by the overexpression of the *MDR1* gene product, P-gp, which acts as an energy-dependent, drug-transport pump with broad substrate specificity [1, 2]. P-gp is a 150–180 kDa phosphoglycoprotein whose phosphate content is increased under a variety of conditions associated with active drug transport [3–10]. Several protein kinases, including PKC and protein kinase A, have been shown to phosphorylate P-gp [11–18], and the activation of PKC and the subsequent phosphorylation of P-gp are associated with decreased drug accumulation and increased drug resistance [10, 12, 13, 18, 19]. We have reported recently that activation of PKC results in the physical association of specific PKC isozymes with P-gp [20]. The natural ligand/receptor interaction that initiates this cascade is unknown.

One characteristic of several MDR cell lines is an increased expression of the EGFR compared with that of the parental line [21–24]. Meyers *et al.* [21] first reported that the increase in EGFR was coincident with the development of MDR in cells selected with either vincristine or actinomycin D in independently derived sublines of mouse and Chinese hamster tumor cells. Increased EGFR was also found in MDR human neuroblastoma and breast carcinoma cells [22–24]. EGFR is a member of a large family of protein tyrosine kinases that play an important role in signal transduction [25]. The activity of the tyrosine kinase domain of the EGFR is controlled by EGF and plays a major role in the regulation of cell proliferation. However, the role that EGFR plays in the MDR phenotype is unclear. Meyers *et al.* [26] suggested that EGF activates protein phosphatase-1 and -2A, and that phospho-P-gp is a substrate for these phosphatases. In the present investigation, we found that EGF stimulated the phosphorylation of P-gp and enhanced drug transport selectively in MDR human breast cancer cells.

MATERIALS AND METHODS

Materials

Human recombinant EGF and monoclonal anti-human EGFR antibody, Mab c11, were purchased from Life Tech-

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† *Abbreviations:* MDR, multidrug resistance or multidrug resistant; P-gp, P-glycoprotein; ECG, epidermal growth factor; EGFR, epidermal growth factor receptor; VBL, vinblastine; IP3, inositol 1,4,5-triphosphate; DAG, diacylglyerol; PLC, phospholipase C; and PKC, protein kinase C.

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nologies, Inc. (Gaithersburg, MD). Anti-P-gp monoclonal antibody, C219, was purchased from Signet Laboratories, Inc. (Dedham, MA). [^{32}P]Orthophosphate and phosphate-deficient RPMI 1640 medium were obtained from ICN Biomedicals (Irvine, CA). Polyclonal antibody mdr (Ab-1), which recognizes human P-gp, was purchased from Oncogene Science (Uniondale, NY). Protein-A Sepharose CL-4B was purchased from Pharmacia (Piscataway, NJ). [^{3}H]VBL (9 Ci/nmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). [^{3}H]*myo*-Inositol, a DAG assay reagents kit, and [γ-^{32}P]ATP were purchased from Amersham (Arlington Heights, IL).

Cell Lines and Cultures

MCF-7/AdrR, and MDR human breast cancer cell line, and MCF-7, the sensitive parental line, were obtained originally from Dr. Kenneth Cowan of the National Cancer Institute (Bethesda, MD) and maintained in RPMI 1640 medium containing 10% (v/v) fetal bovine serum 100 U/mL penicillin and 100 μg/mL streptomycin at 37° in a humidified atmosphere containing 5% CO_2/95% air. Cells were checked routinely and found to be free of contamination by mycoplasma or fungi. Cell lines were discarded after 3 months at which time new cells were obtained from frozen stocks.

Western Blot Analysis of P-gp and EGFR

Plasma membrane proteins were prepared by differential centrifugation as previously described [27]. Proteins were dissolved in sample buffer and separated on 7% (w/v) SDS-polyacrylamide gels [28]. Transfer of proteins to nitrocellulose was performed by the method of Towbin *et al.* [29]. The blots were pre-blocked with 3% (w/v) BSA in 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% (v/v) Tween 20 for 1 hr at room temperature, and then incubated with the anti-P-gp antibody, C219, or anti-human EGFR antibody, Mab c11, diluted in blocking solution overnight at 4°. Detection of immunoreactive proteins was accomplished using horseradish peroxidase-conjugated goat anti-mouse antibodies and the ECL chemiluminescence detection system (Amersham) as per the manufacturers' protocol.

Determination of P-gp Phosphorylation

Four million cells were plated in a 100-mm petri dish in 10 mL RPMI 1640 medium with 10% (v/v) fetal bovine serum. At 90% confluency, cells were rinsed twice with phosphate-free RPMI 1640 and then labeled with 1 mCi [^{32}P]orthophosphate/2 mL phosphate-free RPMI 1640 medium with 0.3% (v/v) fetal bovine serum for 2 hr at 37°. The cells were treated with EGF (500 ng/mL) or vehicle for varying periods of time. Concentration–response experiments showed that 100 ng/mL of EGF was the minimum concentration required to stimulate the phosphorylation of P-gp (data not shown). Radiolabeled lysates were prepared by washing the adherent cells twice with 5 mL of STE buffer [20 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (v/v) aprotinin]. Cells were harvested by scraping into 5 mL of STE buffer and collected by centrifugation (900 *g*) for 10 min, and resuspended in 1 mL RIPA buffer [20 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (w/v) SDS, 10 mM NaF, 2 mM PMSF and 1% (v/v) aprotinin]. The lysates were transferred to an Eppendorf tube and clarified at 16,000 *g* for 30 min at 4°. Proteins were immunoprecipitated from the precleared lysates by incubation with mdr (Ab-1) polyclonal antibody overnight at 4°. The immune complexes were precipitated using protein A-Sepharose, and immunoprecipitated proteins were released by the addition of 50 μL of Laemmli sample buffer and electrophoresed on a 7.5% (w/v) SDS-polyacrylamide gel.

Measurement of IP_3

Five hundred thousand cells were plated in 35-mm dishes and labeled with [^{3}H]*myo*-inositol (3 μCi/mL) in inositol-free medium for 48 hr at 37° After washing, the cells were treated with EGF (500 ng/mL) or vehicle for varying periods of time. The incubations were terminated by the addition of 0.6 mL of 5% (v/v) perchloric acid. Acid extracts were clarified by centrifugation and applied to Bio-Rad AG-X2 ion exchange columns to separate inositol phosphates as previously described [30]. IP_3 was eluted between 0.1 M formic acid/0.4 M ammonium formate and 0.1 M formic acid/0.8 M ammonium formate. A 2-mL portion of each fraction was taken for liquid scintillation counting.

Measurement of DAG

One million cells plated in 60-mm dishes were treated with EGF (500 ng/mL) or vehicle for varying periods of time. The incubations were terminated by aspirating the medium and rapidly washing the cells three times on ice with cold PBS. Samples were extracted with chloroform/methanol, and the chloroform phase was analyzed for DAG by a radioenzymatic assay [31]. This assay employs DAG kinase, which stoichiometrically converts DAG to phosphatidic acid. Following extraction and separation of [^{32}P]phosphatidic acid from [γ-^{32}P]ATP and other ^{32}P-labeled species, the [^{32}P]phosphatidic acid was quantitated by liquid scintillation counting.

Drug Accumulation and Transport

The accumulation and transport of VBL were determined as previously described [12]. Cells were seeded in 24-well plates at a density of 2×10^5 cells/well (1 mL/well) on day 1. On day 2, the growth medium was aspirated and replaced with 0.25 mL of RPMI 1640 containing 25 mM HEPES, pH 7.4, and 50 nM [^{3}H]VBL. After 2 hr at 37°, EGF (500

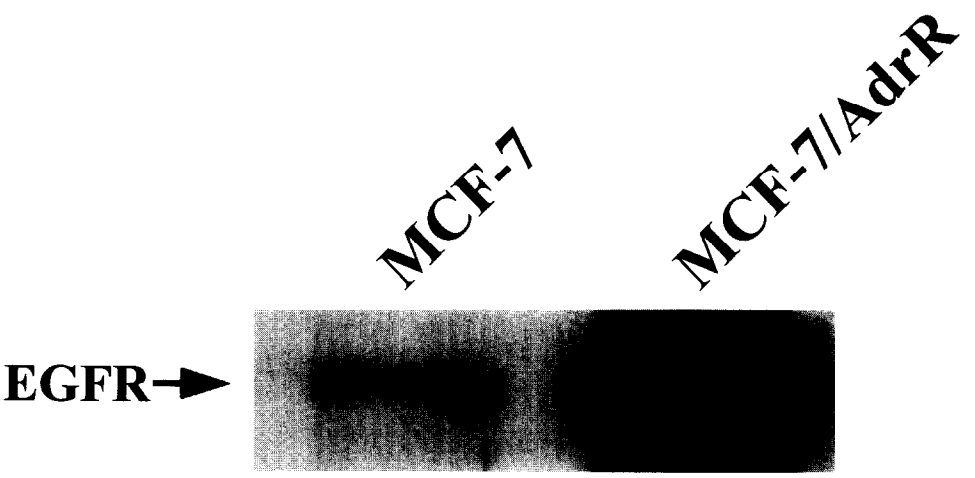

FIG. 1. Expression of EGFR in sensitive and resistant MCF-7 cell lines. Plasma membrane proteins were prepared by differential centrifugation, and identical amounts of membrane proteins (12.5 μg) were resolved using 7% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with a monoclonal anti-human EGFR antibody, Mab c11, as described in Materials and Methods. Results are representative of two similar experiments.

ng/mL) or vehicle was added. After various periods of time, the cells were cooled rapidly and washed twice with 0.5 mL of ice-cold PBS. Cells were solubilized with 0.25 mL of 0.1% (w/v) SDS, and the radioactivity in the samples was determined by scintillation counting. Uptake of VBL was determined by the procedure described above, except that the cells were incubated with EGF or vehicle for 30 min before the addition of VBL.

Drug efflux was determined by loading cells with VBL for 3 hr, then washing cells in VBL-free medium, and measuring the appearance over time of radiolabeled drug in the medium.

RESULTS

Comparison of the Expression of EGFR in Sensitive and Resistant MCF-7 Cells

To confirm the reported overexpression of EGFR in human MDR breast cancer cell lines, we measured the expression of EGFR in both sensitive and resistant MCF-7 cells. Figure 1 demonstrated that MCF-7/AdrR cells expressed 27-fold higher levels of EGFR than the parental MCF-7 line, as determined by western blot analysis of cellular membrane proteins and analysis by densitometry.

Effect of EGF on the Phosphorylation of P-gp in Sensitive and Resistant MCF-7 Cells

To determine the effect of EGF on the phosphorylation of P-gp, cells were treated with 500 ng/mL EGF for varying periods of time, and the phosphorylation of P-gp was measured as described in Materials and Methods. Figure 2 demonstrates that treatment of MCF-7/AdrR cells with EGF for 5, 15, and 30 min increased the phosphorylation of P-gp by 20–50%. The effect was maximal after 5–15 min and began to decrease by 30 min of exposure to the ligand. The content of P-gp, as determined by western blot, did not change after treatment with EGF for 15 min (Fig. 3),

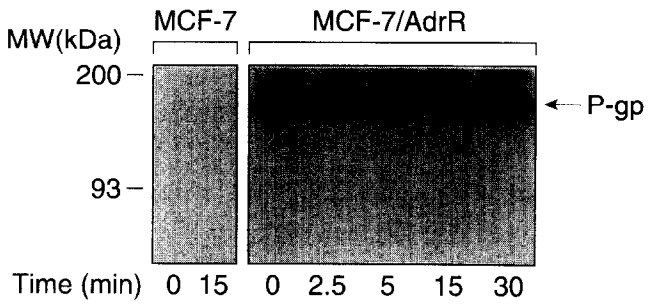

FIG. 2. Effects of EGF on the phosphorylation of P-gp in sensitive and resistant MCF-7 cell lines. MCF-7 and MCF-7/AdrR cells were labeled with [^{32}P]orthophosphate at 37° for 2 hr, and then treated with EGF (500 ng/mL) or vehicle for the indicated periods of time. P-gp was immunoprecipitated from cell lysates using the polyclonal anti-P-gp antibody, mdr (Ab-1). The samples were analyzed by 7.5% SDS–PAGE, as described in Materials and Methods. The protein size markers were ^{14}C-methylated protein standards (mol. wt: 14,300–200,000). Results are representative of three similar experiments.

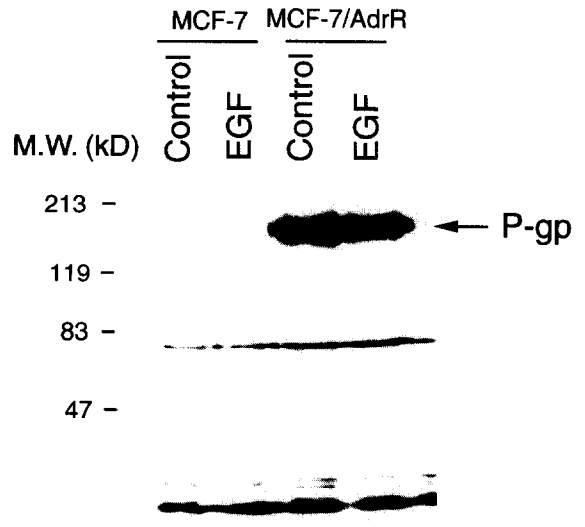

FIG. 3. Effect of EGF on the expression of P-gp in MCF-7/AdrR cells. Cells were treated with EGF (500 ng/mL) or vehicle for 15 min. Membrane proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with the C219 monoclonal antibody, as described in Materials and Methods. Results are representative of two similar experiments.

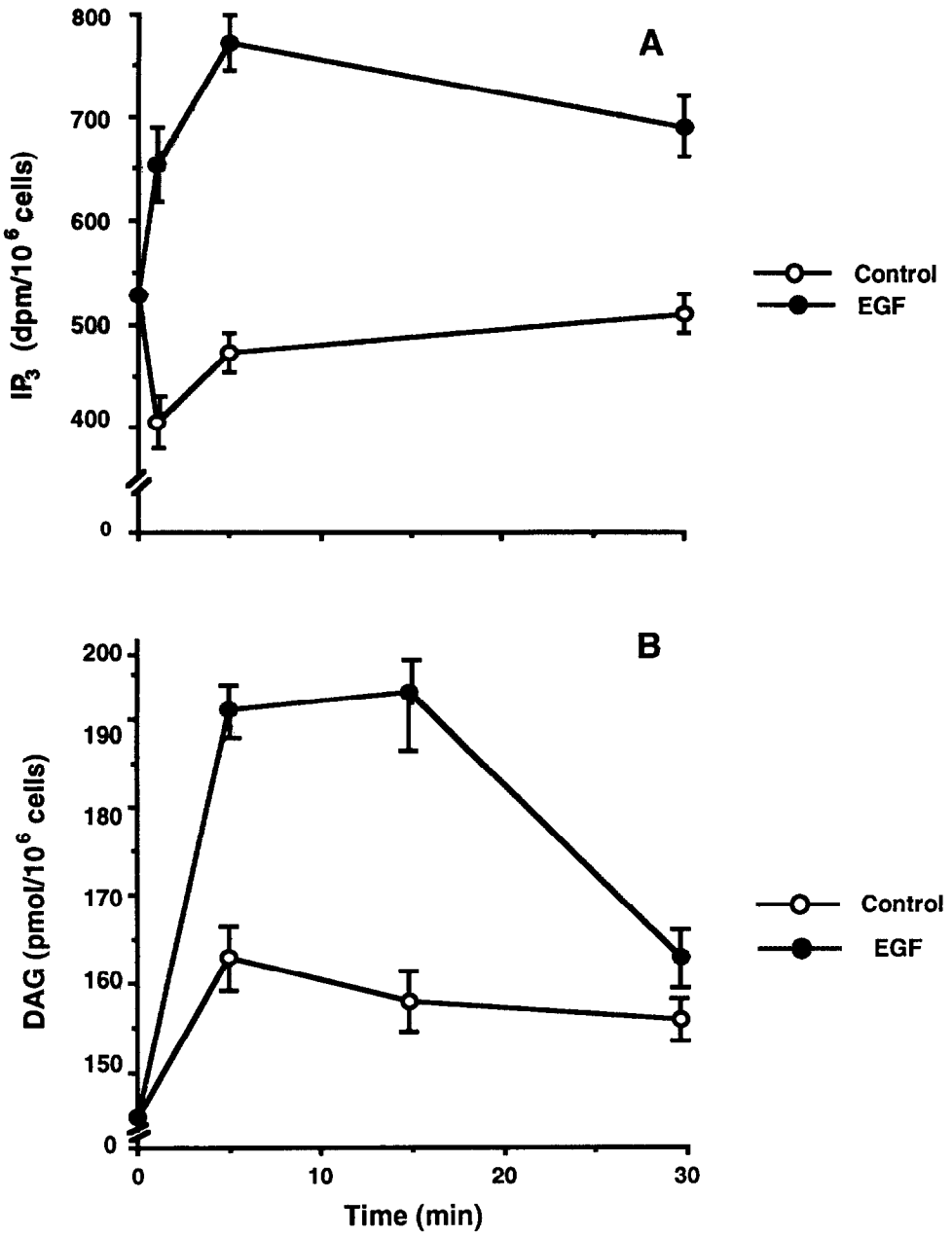

FIG. 4. Effect of EGF on PLC activity (A) and DAG production (B) in MCF-7/AdrR cells. (A) Cells were labeled with [^{3}H]myo-inositol (3 μCi/mL) in inositol-free medium for 48 hr at 37°, and then were treated with EGF (500 ng/mL) or vehicle for the indicated periods of time. The incubations were terminated by adding 0.6 mL of 5% perchloric acid and placing the cultures in ice baths. IP_3 was measured using Bio-Rad AG-X2 ion exchange columns as described in Materials and Methods. Each point represents the mean ± SEM of triplicate experiments. (B) Cells were incubated with 500 ng/mL EGF or vehicle for the indicated periods of time. The reactions were terminated by aspirating the medium and rapidly washing the cells three times with ice-cold PBS. Samples were extracted with chloroform/methanol, and the chloroform phase was analyzed for DAG by a radio-enzymatic assay as described in Materials and Methods. Each point represents the mean ± SEM of triplicate experiments.

suggesting that the increases in phosphorylation of P-gp induced by EGF did not result from increased expression of the protein. In sensitive, parental MCF-7 cells, which do not express P-gp, no phosphorylation of the 180 kDa protein was seen in the presence or absence of EGF (Fig. 2).

Effect of EGF on the Activity of PLC in Resistant MCF-7 Cells

To determine whether activation of PLC was temporarily consistent with the phosphorylation of P-gp following exposure to EGF, we measured the activity of PLC in cells following exposure to EGF. As shown in Fig. 4, treatment of MCF-7/AdrR cells with EGF increased the production of IP_3 (Fig. 4A) and DAG (Fig. 4B) by 5 min of incubation.

Effect of EGF on the Accumulation, Uptake, and Efflux of VBL in Sensitive and Resistant MCF-7 Cells

To investigate the effect of EGF on the function of P-gp, we measured the accumulation, uptake, and efflux of [^{3}H]VBL after a 2-hr incubation with 50 nM [^{3}H]VBL. As shown in Fig. 5, sensitive MCF-7 cells accumulated 40-fold more of the drug than the resistant line (≈12 pmol/10^6 cells vs ≈0.3 pmol/10^6 cells). Figure 5 also demonstrates that EGF produced a 12–23% decrease in the accumulation of VBL in MCF-7/AdrR cells (Fig. 5A), but had no effect on drug accumulation in sensitive MCF-7 cells (Fig. 5B).

Treatment of MCF-7/AdrR cells with EGF decreased the rate of VBL uptake by 20–40% (Fig. 6A). Under identical experimental conditions, EGF had no effect on the uptake of VBL in sensitive MCF-7 cells (Fig. 6B).

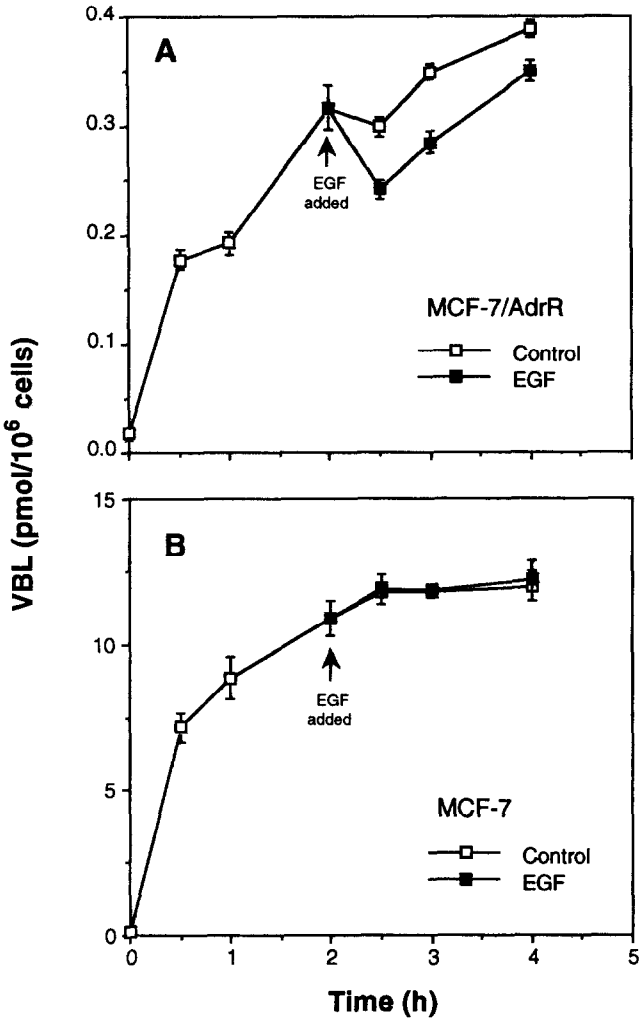

FIG. 5. Effect of EGF on the accumulation of VBL in MCF-7/AdrR (A) and MCF-7 (B) cells. Cells were incubated in medium containing 50 nM [^{3}H]VBL for 2 hr before adding 500 ng/mL EGF or vehicle. Cellular associated VBL was determined at various time points as described in Materials and Methods. Each point represents the mean ± SEM of triplicate experiments.

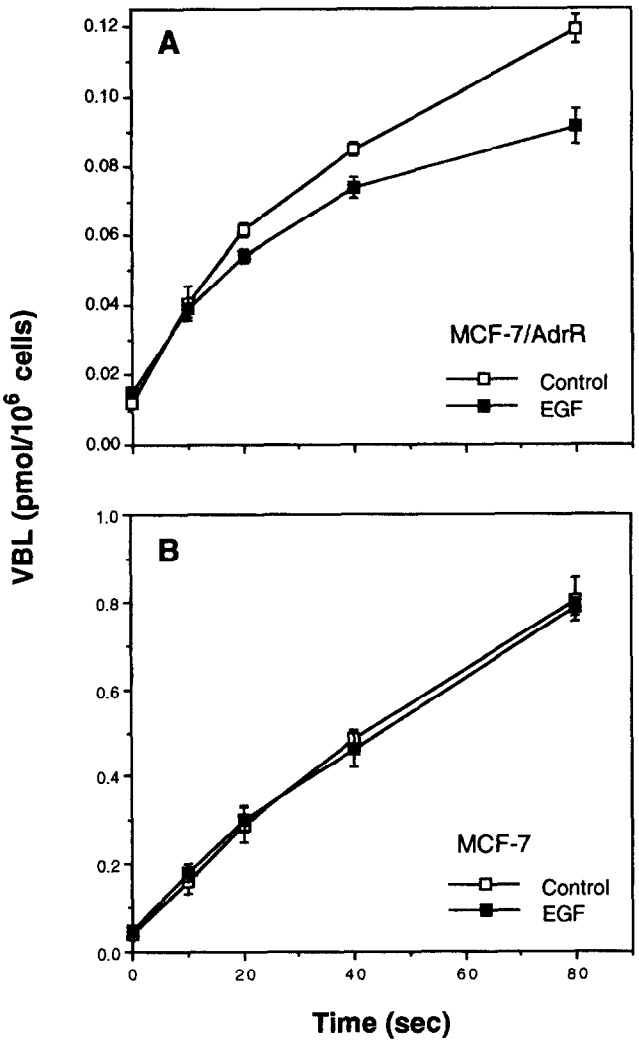

FIG. 6. Effect of EGF on the uptake of VBL in MCF-7/AdrR (A) and MCF-7 (B) cells. Cells were preincubated in medium containing 500 ng/mL EGF or vehicle for 30 min; then [^{3}H]VBL was added to a final concentration of 50 nM. Cellular associated VBL was determined at various time points by rapidly washing the cells twice with ice-cold PBS and solubilizing the cells with 0.25 mL of 0.1% SDS. Radioactivity was determined by scintillation counting. Each point represents the mean ± SEM of triplicate experiments.

To determine the effect of EGF on drug efflux, cells were loaded with VBL for 3 hr, washed, and resuspended in drug-free medium. The effect of EGF on the efflux of VBL was determined by measuring the appearance of [^{3}H]VBL in the medium. Figure 7 demonstrates that EGF increased the efflux of VBL by 25–40% in MCF-7/AdrR cells (Fig. 7A), but had no effect on efflux of the drug from the sensitive cell line (Fig. 7B).

DISCUSSION

The purpose of this study was to investigate the potential link between the often observed increase in both EGFR and P-gp in MDR cancer cell lines of epithelial origin [21–24]. The MDR human breast cancer cell line, MCF-7/AdrR, has been reported previously to have an increased EGFR content [23, 24]. Because the MCF-7 human breast cancer cell lines grown in different laboratories may differ in biological properties [32, 33], we measured the expression of EGFR in both sensitive and resistant MCF-7 cell lines that were used for the current investigations. Figure 1 confirms that the MCF-7/AdrR line overexpresses the EGFR compared with that of the sensitive, parental line.

Activation of the EGFR with EGF produced a rapid (5–15 min) but transient increase in the phosphorylation of P-gp (Fig. 2). This time-course of phosphorylation/dephosphorylation has been observed frequently in EGF-mediated signaling reactions [34–36]. For example, EGF-induced phosphorylation of PLC-II is rapid and maximal after 30-sec incubation with EGF in HER14 cells and disappears after 5 min [35].

The EGFR mediates some of its downstream effects through the activation of PLC by the tyrosine kinase

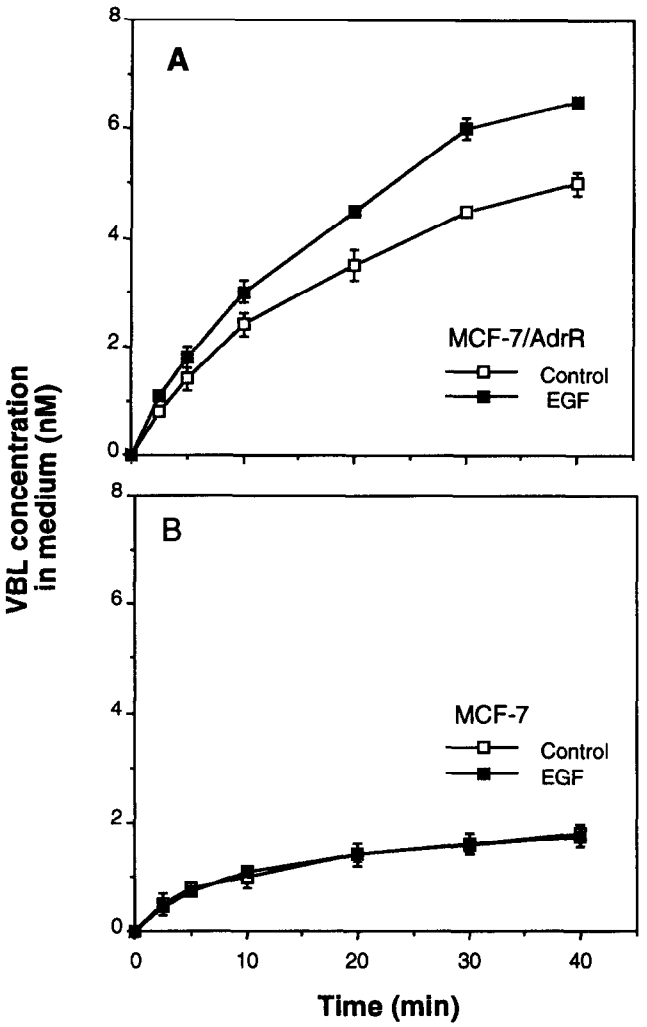

FIG. 7. Effect of EGF on the efflux of VBL in MCF-7/AdrR (A) and MCF-7 (B) cells. Cells were incubated in medium containing 50 nM [^{3}H]VBL for 3 hr and then quickly washed with drug-free medium. EGF (500 ng/mL) or vehicle containing medium was added, and at various time points the content of [^{3}H]VBL in the medium was measured by scintillation counting. Each point represents the mean ± SEM of triplicate experiments.

activity of the EGFR [25, 34, 35]. To determine if this pathway was involved in the phosphorylation of P-gp, we incubated MCF-7/AdrR cells with EGF and measured the activity of PLC. EGF treatment of MCF-7/AdrR cells increased the activity of PLC as measured by the generation of IP_3 and DAG (Fig. 4). Furthermore, the time-course of the activation of PLC ($\leq$2 min) preceded the phosphorylation of P-gp ($\geq$5 min; Fig. 2).

There is mounting evidence that suggests that the phosphorylation of P-gp is mediated by PKC [11–18]. Furthermore, we have proposed that the signaling from membrane to cytosolic PKC is via the activation of PLC and the production of DAG. This results in the translocation of PKC to the membrane and phosphorylation of P-gp [37]. The current studies support this hypothesis. In addition, activation of PLC by other means, such as heat shock or doxorubicin, increases the phosphorylation of P-gp. Furthermore, this effect can be blocked by PLC inhibitors [16]. More recently, we have found, using a coimmunoprecipitation technique, that specific PKC isozymes (PKC-α, -β, -γ, -ϵ, and -θ) form stable complexes with P-gp, whereas PKC-δ, -μ, -ζ, and -λ do not [20].

P-gp is phosphorylated under a variety of experimental conditions. Those associated with increased demands on P-gp function, such as incubation with cytotoxic drugs, are associated with "hyperphosphorylation" of the molecule [4–8, 38, 39]. Yet, it remains uncertain whether phosphorylation *per se* affects P-gp function. For example, Goodfellow *et al.* [40] demonstrated that mutant P-gp lacking consensus phosphorylation sequences continues to function. However, these investigations failed to determine whether the mutant P-gp could increase transport in response to stress. In addition, Ahmad *et al.* [41] found that PKCα could directly increase both the phosphorylation of P-gp and its drug-stimulated ATPase activity. Therefore, phosphorylation of P-gp may not be required for basal transport activity of P-gp, but rather for increased activity following changes in local environment.

EGF produces changes in drug transport that were observed only in MDR cells (Figs. 5–7). The greatest effects were seen on drug uptake (30–40% decrease; Fig. 6) and drug efflux (15–30% increase; Fig. 7). The decrease in initial rate of uptake and the increase in drug efflux are consistent with the model of drug efflux from the plasma membrane proposed by Shalinsky *et al.* [42].

Previous studies by Meyers *et al.* [26] have shown that treatment of MDR cells with EGF activates protein phosphatase-1 and -2A, and thereby stimulates the dephosphorylation of P-gp. They also found that EGF increases phosphorylation of P-gp at certain time points [26]. Here, we demonstrate that EGF initially activated PLC and increased the phosphorylation of P-gp, which was followed by dephosphorylation of the molecule toward baseline values. In spite of the fact that the concentrations of EGF and the cell lines used in these studies were different, it appears that EGF may affect both PLC and protein phosphatases. This may explain why the increased phosphorylation of P-gp was transient in both cases, why the effect of EGF on the function of P-gp was not prolonged, and why the magnitude of changes in the phosphorylation and function of P-gp was only 20–50%.

The formation of DAG, an activator of PKC, may provide an explanation for the phosphorylation of P-gp. For example, PKC activators such as phorbol esters increase the phosphorylation of P-gp and increase drug resistance [13, 19]. Phorbol esters have also been shown to decrease drug accumulation and enhance drug efflux [6, 8, 11, 12, 17]. We have reported previously that heat shock-stimulated phosphorylation of P-gp is mediated by the activation of PLC and PKC [16]. In the current study, we found that EGF activated PLC, increased the phosphorylation of P-gp, decreased drug uptake and accumulation, and increased drug efflux. These results provide evidence that the PLC-

PKC pathway is important in the regulation of the phosphorylation and function of P-gp.

Stimulation of PLC by EGF is a known regulatory mechanism linking the tyrosine kinase activity of EGFR to the phosphatidylinositol-4,5-bisphosphate hydrolysis signaling pathway [35]. The current investigation may help link this pathway to the regulation of P-gp in MDR cells that have increased EGFR, and thus may help elucidate the role that the increased EGFR plays in the MDR phenotype. It is also possible that the EGFR is involved in the transcriptional regulation of the *MDR1* gene. In this regard, Rohlff and Glazer [43] have shown that EGF can stimulate the *MDR1* promoter. Therefore, EGF may regulate P-gp at the transcriptional and post-translational level. This may explain the common occurrence of increased EGFR and P-gp in cell lines and tumors of epithelial origin.

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