Reversal Mechanism of Multidrug Resistance by Verapamil: Direct Binding of Verapamil to P-Glycoprotein on Specific Sites and Transport of Verapamil Outward across the Plasma Membrane of K562/ADM Cells

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ABSTRACT

The calcium channel blocker verapamil has been shown to reverse multidrug resistance (T. Tsuruo et al., Cancer Res. 41: 1967–1972, 1981), but the mechanism of action of this agent has not been fully elucidated. A radioactive photoactive analogue of verapamil, [N-benzyloxy-3,3'-H]-(±)-5-{(3,4-dimethoxyphenethyl)methylaminol-2-(3,4-dimethoxyphenoxy)-2-isopropyl-N-p-azidobenzoylpentylamine, was used to label the plasma membranes of a human myelogenous leukemia cell line (K562), a multidrug-resistant subline selected for resistance to Adriamycin (K562/ADM) and its revertant cell (R1-3). Sodium dodecyl sulfate-polyacrylamide gel electrophoretic fluorograms revealed the presence of an intensely radioactive band with a molecular weight of about 170,000–180,000 in K562/ADM but not from the drug-sensitive parental K562 and revertant R1-3 cells. The M, 170,000–180,000 verapamil acceptor was immunoprecipitated by monoclonal antibody MRK16 specific for P-glycoprotein associated with multidrug resistance, indicating that P-glycoprotein in the plasma membrane is a major target of verapamil in K562/ADM cells. The photolabeling of P-glycoprotein with [N-benzyloxy-3,3'-H]-(±)-5-{(3,4-dimethoxyphenethyl)methylaminol-2-(3,4-dimethoxyphenoxy)-2-isopropyl-N-p-azidobenzoylpentylamine was significantly blocked by other calcium channel blockers, nicardipine and diltiazem, and colchicine, suggesting that the specific binding sites for verapamil on P-glycoprotein are closely related to the binding sites for these calcium channel blockers and antitumor agents. To determine whether verapamil could be a substrate for P-glycoprotein, the cellular accumulation of [3H]verapamil into K562 and K562/ADM was evaluated. The accumulation of [3H]verapamil in the multidrug-resistant cells was 35% of K562 cells and increased when K562/ADM cells were treated with vincristine and nicardipine at 5 μM, indicating that the P-glycoprotein transports verapamil as well as other antitumor agents in the multidrug-resistant cells. These results suggest that verapamil enhances antitumor agent retention through competition for closely related binding sites on P-glycoprotein.

INTRODUCTION

One of the serious obstacles to optimal cancer chemotherapy is the development of drug-resistant tumor cells during treatment. Multidrug resistance is frequently characterized by cross-resistance to functionally and structurally unrelated drugs and by decrease of drug accumulation in resistant cells compared with their drug-sensitive parental cell lines (1, 2). Concomitantly, overexpression of a M, 170,000–180,000 surface membrane glycoprotein (P-glycoprotein) is usually observed with the emergence of multidrug resistance (3–8). Drug-sensitive cells express the multidrug-resistant phenotype after being transfected with the complementary DNA that codes for P-glycoprotein (9–11). The specific binding of Vinca alkaloids for P-glycoprotein was shown using a radioactive photoactive analogue of vinblastine (12, 13). We recently succeeded in purifying P-glycoprotein by means of immunoaffinity chromatography and found that the purified P-glycoprotein has ATPase activity (14) and that the high-affinity binding of vincristine to the plasma membrane of drug-resistant cells is energy dependent (15). In addition P-glycoprotein-containing membrane vesicles prepared from multidrug-resistant cells can transport [3H]vinblastine in a ATP-dependent manner (16). These studies confirm that the P-glycoprotein functions as an energy-dependent efflux pump that is responsible for maintaining the multidrug resistance phenotype.

In 1981, we reported that the calcium channel blocker verapamil inhibited the active drug efflux and restored drug sensitivity in multidrug-resistant cells (17). Various compounds, including calcium channel blockers and calmodulin inhibitors, have been shown to enhance the cytotoxic activity of various antitumor agents (Refs. 18–23; for review see Ref. 24). Verapamil has been reported to be a good inhibitor of the photoaffinity labeling of P-glycoprotein with vinblastine photoanalogue (12, 13) and the vinblastine transport in vesicles from multidrug-resistant cells (16). Recently, direct binding of verapamil on P-glycoprotein was shown using Chinese hamster lung cells resistant to vincristine (25).

In this report, we show the direct binding of verapamil to P-glycoprotein using a verapamil photoanalogue. Furthermore, verapamil was found to be actively transported outward. These findings suggest that verapamil reverses multidrug resistance by competitively inhibiting drug transport.

MATERIALS AND METHODS

Chemicals. DMDI (26) was provided by Eisai Co., Ltd., Tokyo, Japan. N-[benzoate-3,3'-H]-succinimydil 4-azidobenzoate was purchased from NEN Research Products (Boston, MA). [3H]Verapamil (70 Ci/mmol; 99%) was purchased from Amersham Japan, Ltd. The calcium channel blockers were obtained from the following sources: verapamil, from Eisai Co.; and diltiazem, from Tanabe Co., Ltd., Tokyo, Japan. The antitumor agents were obtained from the following sources: Adriamycin, from Kyowa Hakko Co., Ltd., Tokyo, Japan; vincristine, from Shionogi Co., Ltd., Osaka, Japan; and colchicine, from Sigma Chemical Co., St. Louis, MO. All other chemicals were obtained commercially and were of reagent grade.

Cell Culture. The human myelogenous leukemia cell line (K562) was provided by Dr. K. Ezaki, Cancer Chemotherapy Center (Tokyo), and its Adriamycin-resistant subline (K562/ADM) (8) and a revertant subline derived from K562/ADM (27) were established in our laboratory.

Cell lines were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Grand Island Biological Co., Grand Island, NY) and kanamycin (100 μg/ml).

Preparation of Plasma Membrane. Preparation of the plasma membranes was performed according to the previously described method (15). In brief, cells were washed with phosphate-buffered saline (0.15 M NaCl-20 mM sodium phosphate, pH 7.4), suspended at 2 × 108 cells/ml in the hypotonic lysis buffer [10 mM Tris-HCl (pH 7.4)-10 mM NaCl-1.5 mM MgCl2-1 mM dithiothreitol], and incubated for 5 min at 4°C.

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The abbreviations used are: DMDI, (±)-5-{(3,4-dimethoxyphenoxy)ethylamino}-2-(3,4-dimethoxyphenoxy)ethyl-N-p-azidobenzoylpentylamine; [3H]NAB-verapamil, N-[benzoate-3,3'-H]-(±)-5-{(3,4-dimethoxyphenoxy)ethylamino}-2-(3,4-dimethoxyphenoxy)ethyl-N-p-azidobenzoylpentylamine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
in an ice bath. The swollen cells were disrupted with a Dounce homogenizer. The homogenate was then diluted 1:5 with the lysis buffer and centrifuged at 10,000 × g for 10 min to remove the nuclei. The supernatant was layered onto a 35% sucrose solution (10 mM Tris-HCl, pH 7.5) and centrifuged for 60 min at 18,000 × g. The pellet was resuspended in 10 mM Tris-HCl, pH 7.5, and 0.25 mM sucrose. These membrane preparations were stored at −70 °C until use. Protein was determined by the method of Smith et al. (28), using bovine serum albumin as a standard.

Preparation of [3H]NAB-Verapamil. The photoactive analogue, [3H]NAB-verapamil, was prepared from DMDI and N-[benzoyl-3,5-3H]-succinimidyl 4-azidobenzoate (25). All procedures were performed in near total darkness. N-[benzoyl-3,5-3H]-succinimidyl 4-azidobenzoate (1 nmol, 47.7 Ci/mmol) and DMDI (1 μmol) in 1 ml of chloroform containing 5% (v/v) methanol were incubated at 35°C. After 36 h, the reaction mixture was spotted on a 0.2-mm Silica Gel 60 thin layer plate (Merck AG, Darmstadt, Federal Republic of Germany) and [3H]NAB-verapamil was purified by ascending chromatography (solvent system, chloroform:methanol:acetic acid; 100:5:2.5). [3H]NAB-verapamil had an Rf of 0.24. DMDI had an Rf of 0.04 and unreacted succinimidyl 4-azidobenzoate had an Rf of 0.67. [3H]NAB-verapamil was eluted with ethanol and stored in dimethyl sulfoxide at 4°C until use. The purity of the verapamil photoanalogue was confirmed by ascending chromatography. The product gave a single fluorescent spot and no other UV-absorbing spot, and a radioactive spot was obtained on silica gel thin layer chromatography. The reaction yield of [3H]NAB-verapamil was 70% of the starting radioactivity.

Photolabeling of Plasma Membrane. Plasma membranes (50 μg of protein) were photolabeled in 40 mM Tris-HCl buffer (pH 7.2), containing 4% dimethyl sulfoxide and 50 mM [3H]NAB-verapamil in a final volume of 50 μl. The reaction mixture was preincubated for 15 min at 25°C and then irradiated for 15 min with a 400-W mercury 365-nm lamp (Model H400PL; Chiyoda Kohan Co., Ltd., Tokyo, Japan) at a distance of 10 cm at 0°C. Photolabeled membranes were then subjected to SDS-PAGE by the method of Laemmli (29) using gradient gels (4–20%). A total of 12 μg of protein was loaded onto each lane. Covalent incorporation of [3H]NAB-verapamil was detected by fluorography. In brief, the gels were fixed in a mixture of 25% isopropanol and 10% acetic acid, treated with the fluorographic reagent Amplify (Amersham Japan, Ltd.) for 30 min, dried, and then exposed for 14 days at −70°C using Kodak XAR-5 film. Alternatively, quantitation of photolabeling was accomplished by cutting 2-mm areas from the gel, dissolving the gel in 0.25 ml of 30% H2O2 for 1.5 h in a closed scintillation vial at 100°C, and determining radioactivity by liquid scintillation counting.

Immunoprecipitation. The photolabeled membrane (0.25 mg) was suspended in 2 ml of solubilizing buffer [1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate-50 mM Tris-HCl (pH 8.0)-150 mM NaCl-2 mM MgCl2]. The solution was clarified by centrifugation at 22,000 × g for 30 min. Immunoprecipitation was carried out by incubating the membrane extract with 40 μg of monoclonal antibody MRK16, which is specific for P-glycoprotein (30), or normal mouse serum (150 μg) at 4°C for 2 h. Then protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was added. After incubation for 30 min at 4°C, the precipitate were washed 4 times with the solubilizing buffer. The resulting P-glycoprotein antibody-protein A-Sepharose complex was used for SDS-PAGE analysis.

Uptake of [3H]Verapamil. Cell suspensions of K562 and K562/ADM (2 × 106 cells/ml) in the growth medium with 10 mM (N-2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid buffer were incubated at 25°C with [3H]verapamil (2.9 μM; 50 Ci/mmol in the presence (5 μM) or absence of nicardipine (or vincristine). At various intervals, the amount of intracellular [3H]verapamil was determined as described previously (31). In brief, 0.5 ml of the cell suspensions was layered onto a 0.5-ml oil mixture (sodium oil SH550-paraffin liquid, 4:1) and was centrifuged at 13,000 × g for 1 min. The supernatant solution was aspirated, and the sedimented cells were treated with 0.5 M KOH for 2 h at 56°C. The radioactivity in the pelletized cells was determined in an ACS II (Amerham) by liquid scintillation counting.

RESULTS AND DISCUSSION

Photolabeling of Plasma Membranes. K562/ADM was 130-fold more resistant to Adriamycin than the parental cells of K562 and showed marked cross-resistance (640-fold) to vincristine, as has been commonly observed for various multidrug-resistant tumor lines (8). Verapamil enhanced the cytotoxicity of Adriamycin and vincristine for K562/ADM cells. An approximate 30- and 42-fold increase in Adriamycin and vincristine cytotoxicity, respectively, occurred in K562/ADM cells with 6.6 μM verapamil.

The plasma membrane prepared from K562/ADM cells was photolabeled with a photoactive analogue of verapamil, [3H]NAB-verapamil. Following the high-intensity UV irradiation and SDS-PAGE, [3H]NAB-verapamil was incorporated into a M, 170,000–180,000 component (Fig. 1A, Lane 1). Radiolabel incorporation into the M, 170,000–180,000 component represented 4.6% of the initial radioactivity. The M, 170,000–180,000 component was a major target of verapamil in the plasma membrane from multidrug-resistant K562/ADM cells. Another labeled component (M, 33,000) was found in K562 plasma membrane and slightly found in K562/ADM and R1-3 plasma membranes (Fig. 1A). K562/ADM plasma membrane was characterized by the presence of the M, 170,000–180,000 component. The amounts of radiolabeled M, 170,000–180,000 component in K562 and revertant R1-3 cells were 3 and 7%, respectively, of that found in K562/ADM cells.

The incorporation of [3H]NAB-verapamil into a M, 170,000–180,000 component decreased in a dose-dependent manner when verapamil was added, indicating the specificity of the labeling (data not shown). When K562/ADM membranes were photolabeled with [3H]NAB-verapamil in the presence of a 50- and 500-fold excess (in molar basis) of verapamil, the amount of radiolabeling of M, 170,000–180,000 component was reduced by 76% and 87%, respectively. In the presence of 5 × 103-fold excess verapamil, the photolabeling of [3H]NAB-verapamil was completely inhibited.

The M, 170,000–180,000 radiolabeling exhibited a biphasic increase in reaction which is characteristic of mixed specific and nonspecific photolabeling (Fig. 1B). The half-maximal inhibition concentration of verapamil for the photolabeling was 0.8 μM. In the presence of excess verapamil (5 × 103-fold), specific photolabeling was blocked and radiolabeling of M, 170,000–180,000 component increased linearly with a slope parallel to the biphasic curve. The profile of specific [3H]NAB-verapamil photolabeling was obtained by subtracting the nonspecific linear profile from the biphasic profile. The radiolabeling of the M, 170,000–180,000 component reached an apparent saturable maximum of 1.7 pmol of [3H]NAB-verapamil covalently bound per mg of plasma membrane protein.

Identification of a M, 170,000–180,000 Photolabeled Protein. The photolabeled plasma membrane from K562/ADM cells was solubilized with 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate and immunoprecipitated with a monoclonal antibody, MRK16, which has been shown to react with an epitope of P-glycoprotein (30). The radiolabeled M, 170,000–180,000 protein was immunoprecipitated from the solubilized membrane of K562/ADM (Fig. 2). No radioactivity was immunoprecipitated from photolabeled K562/ADM membrane when normal mouse serum was used. These results indicate that the M, 170,000–180,000 verapamil-binding component was identical to the P-glycoprotein associated with the multidrug resistance.

Effects of Other Calcium Channel Blockers and Antitumor Agents on Photolabeling. In the presence of a 5 × 103-fold...
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Fig. 1. Photolabeling of plasma membranes from human myelogenous leukemia K562, K562/ADM, and R1-3 cells with [3H]NAB-verapamil. (A) Plasma membranes (1 mg/ml) were photolabeled with 50 nM [3H]NAB-verapamil. SDS-PAGE fluorograms of photolabeled plasma membranes (12 ng of protein) of K562/ADM (Lane 1), K562 (Lane 2), and R1-3 (Lane 3) with [3H]NAB-verapamil. (B) Photolabeling of the M, 170,000-180,000 component in K562 plasma membrane (12 µg of protein) with increasing [3H]NAB-verapamil concentrations. Plasma membrane and different concentrations of [3H]NAB-verapamil with and without 250 µM verapamil were photolabeled for 15 min and analyzed by SDS-PAGE. Net specific photolabeling was calculated by subtracting the radioactivity incorporated in the presence (○) of verapamil from the radioactivity incorporated in the absence (■) of verapamil. Each point represents the mean radioactivity (n = 3) minus average base line radioactivity ± SD (bars).

excess of other calcium channel blockers, nicardipine, or diltiazem, the radiolabeling of P-glycoprotein was completely inhibited (Fig. 3A) as has been observed with verapamil, indicating that specific binding sites for verapamil are identical to those of other calcium channel blockers, such as nicardipine and diltiazem. Vincristine, Adriamycin, and colchicine were also effective inhibitors of the photolabeling. However, in the presence of a 5 × 10^-3-fold excess of Adriamycin, vincristine, and colchicine, the amount of radiolabeling was reduced by 67, 78, and 50%, respectively (Fig. 3B). In the presence of a 50-fold excess of Adriamycin, vincristine, and colchicine, the radiolabeling was reduced by 30, 65, and 35%, respectively. Camptothecin, which is effective against multidrug-resistant cells (32), had no inhibitory effect on the photolabeling (data not shown). These results suggest that the specific binding sites for the photolabeled analogue of verapamil might not necessarily be identical but closely related to the binding sites for various antitumor agents, such as Adriamycin, vincristine, and colchicine.

Uptake of [3H]Verapamil and Effects of Calcium Channel Blockers and Antitumor Agents. Previously, we demonstrated that vincristine and daunomycin uptakes into K562 and K562/ADM cells were very different (8) and that verapamil greatly enhanced the cellular accumulation of vincristine and daunomycin in multidrug-resistant tumor cells (17-19). Verapamil inhibits the active drug efflux and restored drug sensitivity in multidrug-resistant cells. To determine whether verapamil could be a substrate for the outward transport system of antitumor agents, the cellular accumulation of [3H]verapamil into K562 and K562/ADM was evaluated (Fig. 4). The internal concentration of [3H]verapamil was saturable 30 min after incubation. There was a 3-fold decrease in the intracellular accumulation of [3H]verapamil in K562/ADM cells as compared with K562 cells. This defect in intracellular [3H]verapamil accumulation may account for the existence of active efflux of the drug across the plasma membrane in drug-resistant cell.

Cellular uptake of [3H]verapamil was examined in the presence of the antitumor agent vincristine and the calcium channel blocker nicardipine (Fig. 4). Vincristine, at 5 µM, increased the cellular accumulation of verapamil in K562/ADM cells, indicating that verapamil was transported through an outward carrier identical to that of vincristine. The enhancement of verapamil accumulation at 5 µM nicardipine was more prominent than that at 5 µM vincristine.

Reversal Mechanism of Multidrug Resistance by Verapamil. In order to prove the competitive inhibition of the candidate compound on the drug transport system through P-glycoprotein, two conditions must be satisfied: (a) a direct evidence of binding of the compound to the P-glycoprotein must be ob-
However, it was suggested that dihydropyridine, nitrendipine has been shown to photolabel the P-glycoprotein (36). Drug transport through P-glycoprotein. The consumption was induced by verapamil in multidrug-resistant cells (33). In addition, our present findings are in good agreement with the previous reports showing that verapamil accumulated; (b) the compound must be transported outside the plasma membrane (34).

Therefore dihydropyridine calcium channel blockers might have different reversal mechanism of multidrug resistance. Our present experiments have shown that P-glycoprotein could recognize verapamil as a substrate as well as antitumor agents such as anthracyclines and vincristine. It is of interest that P-glycoprotein can recognize various drug molecules with different structures. Determination of binding sequences for verapamil is a key to elucidating the drug transport mechanism of P-glycoprotein.

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