Identification of the Multidrug Resistance-related Membrane Glycoprotein as an Acceptor for Calcium Channel Blockers

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A radioactive photoactive dihydropyridine calcium channel blocker, [3H]azidopine, was used to photaffinity label plasma membranes of multidrug-resistant Chinese hamster lung cells selected for resistance to vincristine (DC-3F/VCRd-5L) or actinomycin D (DC-3F/ADX). Sodium dodecyl sulfate-polyacrylamide gel electrophoretic fluorograms revealed the presence of an intensely radiolabeled 150–180-kDa doublet in the membranes from drug-resistant but not from the drug-sensitive parental (DC-3F) cells. A similar radiolabeled doublet was barely detected in a drug-sensitive partial revertant (DC-3F/ADX-U) cell line. The 150–180-kDa doublet exhibited a specific half-maximal saturable competitive blocking of specific photolabeling with [3H]azidopine. The dihydropyridine binding specificity was established by competitive blocking of specific photolabeling with nonradioactive azidopine as well as with nonphotoactive calcium channel blockers nifedipine, nitrendipine, and nifedipine. In addition, [3H]azidopine photolabeling was blocked by verapamil and diltiazem but was stimulated by excess prenylamine and bepridil suggesting a cross-specificity for up to four different classes of calcium channel blockers. The 150–180-kDa calcium channel blocker acceptor co-electrophoresed exactly with the 150–180-kDa surface membrane glycoprotein (gp150–180 or P-glycoprotein) vinca alkaloid acceptor from multidrug-resistant cells and was immunoprecipitated by polyclonal antibody recognizing gp150–180. [3H]Azidopine photolabeling of the 150–180-kDa component in the presence of excess vinblastine was reduced over 90%, confirming the identity or close relationship of the calcium channel blocker acceptor and the gp150–180 vinca alkaloid acceptor. The [3H]azidopine photolabeling of gp150–180 also was reduced by excess actinomycin D, adriamycin, or colchicine, demonstrating a broad gp150–180 drug recognition capacity. The ability of gp150–180 to recognize multiple natural product cytotoxic drugs as well as calcium channel blockers suggests a direct function for gp150–180 in the multidrug resistance phenomenon and a role in the circumvention of that resistance by calcium channel blockers.

Multidrug resistance is frequently characterized by diminished drug accumulation in resistant cells compared to their drug-sensitive parental cells (1–7). Concomitant overexpression of a 150–180-kDa surface membrane glycoprotein (gp150–180 or P-glycoprotein) usually correlates with multidrug resistance (8–10). We recently reported that gp150–180 from multidrug-resistant Chinese hamster lung (11) and human carcinoma (12) cell lines specifically binds Vinca alkaloids. This prominent membrane glycoprotein can thus serve as a specific cellular acceptor for at least one of the classes of drugs most commonly associated with the multidrug-resistant phenotype and thus may play a direct functional role in cellular resistance to this class of drugs.

A number of recent reports have described the ability of various agents including calcium channel blockers to partially reverse multidrug resistance, presumably by increasing cellular drug accumulation (13–23). The proposed active outward transport of one such phenylalkylamine calcium channel blocker, verapamil, suggests that this particular inhibitor may increase intracellular drug accumulation by serving as a competitive substrate for the outward drug transporter of resistant cells (19, 24). This suggestion is supported by the ability of verapamil to block vinblastine-specific photolabeling of gp150–180 (12, 25).

In this report, we have used a radioactive photoactive analogue of the dihydropyridine class of calcium channel blockers to directly identify a 150–180-kDa membrane protein as a specific acceptor of calcium channel blockers in multidrug-resistant cells. In addition, we provide evidence that this calcium channel blocker acceptor exhibits cross-reactivity with several different classes of natural product cytotoxic drugs. Our data indicate that this protein is closely related or identical to the gp150–180 Vinca alkaloid acceptor (11) and suggests that gp150–180 is not only instrumental in the maintenance of the multidrug resistance phenotype but that it also plays a direct role in the reversal of multidrug resistance by various calcium channel blockers.

MATERIALS AND METHODS

The radioactive photoactive dihydropyridine calcium channel blocker (26), 2,6-dimethyl-[4-(2'-trifluoromethyl)phenyl]-1,4-dihydropyridine-3,5-dicarboxylic acid, ethyl, (N-4'–azido[3', 5', 3H]benzoylamo)ethyl)dier (3H)azidopine (44 Ci/mmol), was purchased from Amersham Corp. Nonradioactive azidopine was a gift from New England Nuclear. Vinblastine, actinomycin D, adriamycin, and methotrexate were obtained from the Drug Synthesis and Chemistry Division, National Cancer Institute, CA28595 and CA08748. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: gp150–180, 150–180-kDa surface membrane glycoprotein; [3H]azidopine, 2,6-dimethyl-[4-(2'-trifluoromethyl)phenyl]-1,4-dihydropyridine-3,5-dicarboxylic acid, ethyl, (N-4'–azido[3', 5', 3H]benzoylamino)ethyl)diester; SDS, sodium dodecyl sulfate; PAGS, polyacrylamide gel electrophoresis.
Calcium Channel Blocker Acceptor in Multidrug-resistant Cells

Branch, National Cancer Institute, Bethesda, MD. Colchicine, verapamil, nifedipine, diltiazem, prenylamine, and bepridil were purchased from Sigma. Nimodipine and nifedipine were supplied by Miles Pharmaceuticals, New Haven, CT.

Vinblastine-resistant (DC-3F/VCRd-5L) (2750-fold resistant) and actinomycin D-resistant (DC-3F/ADX) (2450-fold resistant) variants were derived from drug-sensitive Chinese hamster lung (DC-3F) cells and cultured as described previously (27, 28). The partial revertant (DC-3F/ADX-U) (30-fold resistant to actinomycin D) was derived from DC-3F/ADX cells grown in the absence of drug (28). The resistant cells expressed multidrug resistance to a number of structurally and mechanistically unrelated natural product cytotoxic agents. Purified plasma membranes were prepared by sucrose density gradient centrifugation as previously (9, 29) and membrane vesicles were prepared by the nitrogen cavitation and differential centrifugal procedure of Lever (30). The basic photolabeling patterns were the same in both purified plasma membranes or membrane vesicles. Protein concentrations were determined by the procedure of Lowry et al. (31).

Immunoprecipitations were performed as previously described (11) with rabbit polyclonal antibody specific for multidrug-resistant gp150-180 (32). Nonimmune rabbit serum was used as a control.

Purified plasma membranes or membrane vesicles (5–25 μg of protein) were photolabeled in 40 mM potassium phosphate buffer (pH 7.0) containing 10 μM CaCl2, 4% dimethyl sulfoxide, and 0.050–0.756 μM [3H]azidopine in a final volume of 0.050 ml. This mixture was preincubated for 1 h at 25 °C in the absence or presence of nonradioactive competing ligand and then irradiated for 20 min with a UV lamp equipped with two 15-W self-filtering 302-nm lamps (Model XX-15), Ultra-Violet Products Inc., San Gabriel, CA (11). Trypan blue viable (>90%) cell suspensions (3–5 × 106 cells) were photolabeled exactly as described above except that the buffer was 7 mM potassium phosphate (pH 7.4) containing 0.15 M NaCl. The absence of physical screening of incident UV irradiation was established by demonstrating the complete photofysis of [3H]azidopine in the presence of the same concentrations of unlabeled calcium channel blockers or natural product drugs but in the absence of protein. In this analysis, [3H]azidopine was quantitated by chromatography of the photolyzed mixtures on silica gel thin layer plates (E. Merck, Darmstadt, Germany) (ethyl acetate/methanol) (90:10, v/v), scraping the silica gel every 1 cm, and determining radioactivity by liquid scintillation counting. Photolabeled membranes were analyzed by 5–15% SDS-PAGE containing 4.5 M urea and by fluorography (11). Quantitation of radioactivity was accomplished by cutting appropriate areas from the gel, removing the paper backing, dissolving the gel in 0.5 ml of 30% H2O2 for 1 h in a closed scintillation vial at 100 °C, and determining radioactivity by liquid scintillation counting. The absence of an inner filter screening of incident light in Fig. 2 was verified by the constant radiolabeling of a prominent minor background 50–55-kDa component in the presence of increasing concentrations of competitors.

RESULTS AND DISCUSSION

Plasma membranes from drug-sensitive Chinese hamster lung (DC-3F) cells, from multidrug-resistant variants independently selected for resistance to vincristine (DC-3F/VCRd-5L) or actinomycin D (DC-3F/ADX) and from a revertant (DC-3F/ADX-U) of the DC-3F/ADX variant, were photoaffinity labeled with the tritiated photoactive analogue of the dihydropyridine calcium channel blocker, [3H]azidopine. Following SDS-PAGE, fluorography revealed a prominent high molecular weight radiolabeled doublet (150–180 kDa) in the membranes from DC-3F/ADX resistant cells (Fig. 1, lanes 2 and 5) and from DC-3F/VCRd-5L-resistant cells (Fig. 3, lane 2) but not from DC-3F drug-sensitive cells (Figs. 1 and 3, lanes 1). Radiolabeling of the membranes from DC-3F/ADX-U revertant cells (Fig. 1, lane 3) was markedly reduced compared to both drug-resistant variants. Radiolabel incorporation into the 150–180-kDa doublet represented 3–4% of the initial radioactivity and about 40% of the total radiolabeled macromolecular (>10 kDa) material. This corresponded to a Coomassie Blue-stained doublet accounting for 3% of the total DC-3F/ADX membrane protein as determined by densitometry (Fig. 1, lane 4). Identical labeling patterns were obtained when [3H]azidopine was used to photolabel vialable DC-3F, DC-3F/ADX, and DC-3F/ADX-U cells in vitro (data not shown).

The [3H]azidopine photolabeling specificity was established by performing the experiments in the presence of ligands representative of four different classes of structurally distinct calcium channel blockers (i.e. dihydropyridine, phenylalkylamine, benzoiazepine, and diphenylalkylamine). In the presence of a 200-fold molar excess of the dihydropyridine calcium channel blockers, nimodipine, nifedipine, nitrendipine, or nifedipine, radiolabeling of the 150–180-kDa doublet was reduced by 80, 67, or 38%, respectively (Fig. 1, lanes 6–8). Moderate inhibition of 150–180-kDa radiolabeling also was obtained when photolabeling was performed in the presence of a 200-fold molar excess of the phenylalkylamine, verapamil (43%) (Fig. 1, lane 9), or the benzoiazepine, diltiazem (31%) (Fig. 1, lane 10). However, no inhibition was observed when the experiments were carried out in the presence of the diphenylalkylamine, prenylamine, or another benzoiazepine analogue, bepridil (Fig. 1, lanes 11 and 12, respectively). In the experiment shown in Fig. 1, the presence of prenylamine or bepridil actually increased 150–180 kDa radiolabeling 31–38%. In other experiments using DC-3F/VCRd-5L membranes, these latter calcium channel blockers stimulated 150–180-kDa radiolabeling as much as 91% (data not shown). Background labeling was not appreciably affected except for diltiazem, prenylamine, and bepridil. However, in the case of the latter two competitors, this was associated with increased rather than decreased 150–180-kDa radiolabeling. The observed inhibition of 150–180-kDa photolabeling was not the result of blockade of incidental activation light by UV absorbing compounds, because UV irradiation under identical conditions, except for the absence of protein, photoactivated 100% of the [3H]azidopine regardless of the presence of competitors. These results confirm the presence in multidrug-resistant cells of a 150–180-kDa acceptor for dihydropyridine calcium channel blockers which has a cross-specificity for...
analouges from at least two additional classes of calcium channel blockers, namely verapamil and diltiazem. Furthermore, the apparent stimulatory effect of prenylamine on 150-180-kDa radiolabeling suggests a further cross-specificity for the diphenyalkylamine class of calcium channel blockers which may exert a positive cooperative effect on \[^3H\]azidopine photolabeling via a separate 150-180-kDa doublet binding site. Alternatively the apparent stimulatory effects could be mediated by distinct macromolecular calcium channel blocker acceptors. It is possible also that the increased 150-180-kDa photolabeling results from an increased concentration of available \[^3H\]azidopine due to displacement from nonspecific sites on background proteins.

The photolabeling specificity was characterized in more detail by photolabeling DC-3F/ADX membrane vesicles in the presence of increasing concentrations of \[^3H\]azidopine. Under these conditions, the 150-180-kDa radiolabeling exhibited a biphasic increase in radiolabeling which is characteristic of mixed specific and nonspecific photolabeling (Fig. 2A). In the presence of excess nimodipine, specific photolabeling was blocked and radiolabeling of the 150-180-kDa doublet increased linearly with a slope parallel to the terminal nonspecific linear portion of the biphasic curve. The specific dihydropyridine photolabeling was obtained by subtracting the nonspecific linear curve from the biphasic profile. The resulting apparent hyperbolic curve exhibited a half-maximal saturable photolabeling of 1.07 X 10^{-7} M \[^3H\]azidopine as determined by reciprocal analysis.

At a fixed saturable \[^3H\]azidopine concentration (i.e. 7.56 X 10^{-7} M as indicated by the arrow in Fig. 2A), an equal concentration of nonradioactive azidopine reduced specific 150-180-kDa photolabeling by about 50%. By comparison, a 6-fold excess of the nonphotoactive dihydropyridine, nimodipine, was required to reduce specific photolabeling by 50%. Furthermore, increasing concentrations of nimodipine gradually reduced radiolabeling to a limiting minimum of 38% of the total photolabeling in the absence of competitor (Fig. 2B). The proportion of radiolabeling not blocked (38%) corresponded closely to the percentage of total nonspecific radiolabeling (40%) found in the saturation photolabeling experiments (Fig. 2A). In contrast, increasing concentrations of prenylamine stimulated \[^3H\]azidopine photolabeling of the 150-180-kDa doublet by about 40% and the metabolic inhibitor, methotrexate, had no affect up to a 200-fold molar excess (Fig. 2B).

These observations establish the presence of a 150-180-kDa calcium channel blocker acceptor in multidrug-resistant cells. The dramatically increased level of this acceptor in drug-resistant cells compared to drug-sensitive parental and revertant cells is analogous to our earlier identification of a similarly increased gp150-180 Vinca alkaloid acceptor in the same cell lines (11). In addition, the \[^3H\]azidopine photolabeled doublet with a major band at 180 kDa and a minor one at 150 kDa co-electrophoresed exactly with the gp150-180 doublet visualized by vinblastine photolabeling. When DC-3F/CRd-5L membranes were \[^3H\]azidopine photolabeled in the presence of a 200-fold molar excess of vinblastine, the 150-180-kDa radiolabeling was reduced by 92% suggesting a Vinca alkaloid specificity for the calcium channel blocker acceptor (25). Overall, our results indicate that there is an identity or close relationship of the calcium channel blocker acceptor and the gp150-180 Vinca alkaloid acceptor. The data also demonstrate that the 150-180-kDa calcium channel blocker acceptor of cells selected with vincristine interacts with four drugs to which these cells are known to display multidrug resistance (29). In contrast, the antimetabolite, methotrexate, to which the cells are sensitive, does not block photolabeling (lane 7). In data to be reported elsewhere, we have observed that photolabeling with the vinblastine photoactive analogue in the presence of the same competitors used in Fig. 2 gave nearly identical competition patterns which confirms the multidrug specificity of the gp150-180 Vinca alkaloid acceptor (26). Overall, our results provide evidence that gp150-180 recognizes several calcium channel blockers as well as cytotoxic drugs. However, the present approach does not allow a clear distinction between the possibility of a single multidrug binding site, or multiple drug binding sites that are allosterically linked or associated in some other way.
Calcium Channel Blocker Acceptor in Multidrug-resistant Cells

The immunocross-reactivity of the 150–180-kDa calcium channel blocker acceptor and the gp150–180 Vinca alkaloid acceptor was established with a polyclonal antibody which has been shown to cross-react with the gp150–180 from several multidrug-resistant cell lines and the P-glycoprotein from CHC5 colchicine-resistant Chinese hamster ovary cells (8, 32, 33). Membranes from parental cells and from the DC-3F/VCRd-5L drug-resistant variant were photoaffinity labeled with [3H]azidopine, detergent-solubilized, and immunoprecipitated with a polyclonal antibody recognizing gp150–180 (Fig. 4). The radiolabeled 150–180-kDa doublet was immunoprecipitated only from membrane extracts from drug-resistant cells (lane 2). Photolabeling in the presence of 70 μM vinblastine reduced the radioactivity in the immunoprecipitate by 85% (lane 3). No radioactivity was immunoprecipitated from photolabeled parental DC-3F membrane extracts (lane 5) nor from photolabeled drug-resistant membrane extracts using nonimmune serum (lane 4).

Our experiments provide the first direct evidence identifying gp150–180 as a calcium channel blocker acceptor in multidrug-resistant Chinese hamster lung cells. These data suggest that this membrane glycoprotein participates in the process by which calcium channel blockers counteract multidrug resistance. Although the molecular mechanism(s) for this phenomenon has yet to be defined, we speculate that gp150–180 mediates resistance by binding drugs and controlling their membrane transport. It is possible, for example, that gp150–180 may function as a component of an active drug-efflux pump (34). Alternatively, the increased absorptive endocytosis reported in drug-resistant cells (35) as well as the potentiation of the sensitivity of the cells to cytotoxic agents in the presence of lysosomotropic agents (36, 37) support the proposal that gp150–180 could function as a drug carrier during active membrane recycling or vesicle-mediated outward extrusion processes. Both models are compatible with the concept that calcium channel blockers counteract multidrug resistance by competing for a common drug acceptor (19, 24).

Our experiments demonstrate that gp150–180 can serve as an acceptor of calcium channel blockers in multidrug-resistant cells and thus there exists a possible relationship of gp150–180 to the calcium channel proteins previously identified in various excitable tissues (38). For example, the rabbit skeletal muscle calcium channel has a molecular mass of 180 kDa and has multiple allosterically linked drug binding sites (38, 39). However, various other normal membrane components such as the sodium channel have similar molecular weights (40), and it is known that calcium channel blockers interact with a number of cellular components other than the calcium channel (38). Our experiments also indicate that gp150–180 is different from the 180-kDa calcium channel in purified rabbit skeletal muscle T-tubules since photolabeling of these T-tubules with [3H]azidopine is not competed by vinblastine nor is a 180-kDa component photolabeled in T-tubules with the photoactive vinblastine analogue.2

Recent data on the probable gene structures of mouse, human, and hamster P-glycoproteins suggest that gp150–180 bears a strong homology with bacterial transport proteins (41–43). Such an identity is consistent also with the ability of

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the calcium channel blocker, verapamil, to potentiate the mutagenicity of anticancer drugs in bacteria and yeast possibly by increased drug retention (44). If this analogy is correct, then gp150-180 may normally function in cellular detoxification as a conserved drug-efflux transporter. It is possible also that gp150-180 fortuitously binds a number of different heterocyclic aromatic compounds and thus mediates multidrug resistance in addition to, but essentially independent of, its normal cellular function. In either case, the multidrug binding capacity of gp150-180 suggests that there is a direct functional role for this protein both in the establishment and maintenance of drug resistance as well as in the circumvention of resistance by calcium channel blockers.

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