AF may induce translocation of PKC by elevating cAMP [17], by analogy with induction of translocation of PKC to nuclear fractions by cAMP in mouse lymphocytes [18]. Alternatively, AF and metals which are known to interact with thiols [19], may act via the cysteine-rich site in the regulatory domain of PKC [20]. Metals have an affinity for the cytoskeleton [21], a region rich in sulphydryls.

In summary, AF, which modulates PKC-dependent events *in vitro*, synergizes with the phorbol ester PDBu, a specific ligand of PKC, in causing translocation of PKC from cytosol to the particulate subcellular fraction. This translocation is accompanied by increased affinity of binding of phorbol ester. Translocation induced in the presence of AF, unlike that induced by phorbol ester alone, results in attachment of PKC to a detergent-insoluble compartment. In this state PKC is not subject to normal down-regulation. Studies are now needed to show whether translocation of PKC to particular subcellular compartments is mediated by particular intracellular messengers.

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Azidopine photoaffinity labeling of multidrug resistance-associated glycoproteins

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The problem of developing effective cancer chemotherapeutic regimens in humans in the presence of drug-resistant tumor cells is being approached by studying model systems in tissue culture. Mammalian cells selected for resistance to drugs often display the multidrug resistance (MDR*) phenotype which includes (1) cross-resistance to unrelated drugs, (2) a net decrease in drug accumulation, and (3) the overexpression of a MDR-associated glycoprotein (MDRG), also known as P-glycoprotein, that is found in the plasma membrane [1]. Calcium antagonists such as verapamil can partially reverse MDR by increasing intracellular drug levels [2–8]. Verapamil specifically inhibits the binding of vinblastine and its photoactive analog to the MDRG, thereby suggesting that it interacts with the MDRG [9–12].

MDR cell lines derived from the murine cell, J774.2, have been selected in our laboratory for resistance to colchicine (CLC), vinblastine (VBL) or taxol (TAX) [13, 14]. The MDRGs observed by SDS–PAGE in the CLC- and VBL-resistant cells have an approximate M_r of 130–150 kDa, whereas the MDRG from the TAX-resistant cells migrates as a doublet in a similar molecular weight range [15, 16].

The arylazide 1,4-dihydropyridine, azidopine, is a calcium antagonist which photolabels calcium channels [17]. [^{3}H]Azidopine has the advantages of being relatively stable in the absence of UV light and capable of forming covalent bonds via nitrene intermediates upon UV irradiation. In

* Abbreviations: MDR, multidrug resistance; MDRG, multidrug resistance glycoprotein; CLC, colchicine; VBL, vinblastine; TAX, taxol; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone.

this report we demonstrate that [^{3}H]azidopine can specifically photolabel the MDRG.

Materials and methods

Materials and cells. [^{3}H]Azidopine (54 Ci/mmol) was purchased from the Amersham Corp. Taxol was obtained from the National Cancer Institute. Drug-resistant cell lines derived from the macrophage-like cell line, J774.2, were isolated and maintained as described [13]. Cell lines maintained in either 100 μM CLC (J7.C1-100), 1 μM VBL (J7.V1-1), 3 μM VBL (J7.V1-3), or 50 μM TAX (J7.T1-50) were 2500-, 1000-, 3000- and 830-fold resistant to the selecting drugs respectively.

Preparation and photoaffinity labeling of microsomal membranes. Confluent cells were homogenized in 10 mM Tris–HCl, pH 7.4, 250 mM sucrose, 0.1 mM PMSF and centrifuged at 3000 *g*. The supernatant fraction was centrifuged at 100,000 *g* for 1 hr. The pellet was resuspended (1 μg/μl) and photolabeled in 50 mM Tris–HCl, pH 7.4, containing protease inhibitors (100 units/ml trasylol, 1 μg/ml pepstatin A, and 30 μM leupeptin) and 50 nM [^{3}H]azidopine. The mixture was preincubated for 1 hr at 25° and then irradiated for 10 min at 4° with a UV lamp (254 nm) at a distance of 8 cm. Photolabeled membranes were analyzed by SDS–PAGE on 5–12% gradient gels [18] and detected by fluorography after 48 hr.

Analysis of the tryptic peptides of [^{3}H]*azidopine-labeled MDRG by HPLC.* The [^{3}H]azidopine-labeled MDRG from J7.V1-1 cells was excised from gels and washed with a solution of 85% acetone, 5% acetic acid and 5% triethylamine. The gel slice was digested at 37° with 50–250 μg/ml TPCK-treated trypsin (Sigma) in 50 mM NH_4HCO_3 (pH 8.0). After 16 hr, a second addition of trypsin was made and the digestion continued for 4 hr. The digested polypeptides were lyophilized, dissolved in 200 μl of 1% trifluoroacetic acid, and subjected to analysis by HPLC on an Altex Ultrasphere ODS reversed phase column (4.6 × 250 mm) and equilibrated with 0.1% trifluoroacetic acid. The digested peptides were eluted using a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid over a period of 70 min. Fractions (0.5 ml/30 sec) were collected and counted.

Results and discussion

(±)-[^{3}H]Azidopine was used to photolabel the membrane proteins of our parental and drug-resistant cell lines. A major high molecular weight radiolabeled protein (130–150 kDa) was seen in membranes from J7.V1-1, J7.V1-3 and J7.C1-100 by fluorography after SDS–PAGE (Figs. 1 and 2). This radiolabeled protein was specifically immunoprecipitated [19] by a polyclonal antibody prepared against the MDRG isolated from J7.V1-1 cells [15] (Fig. 1). In

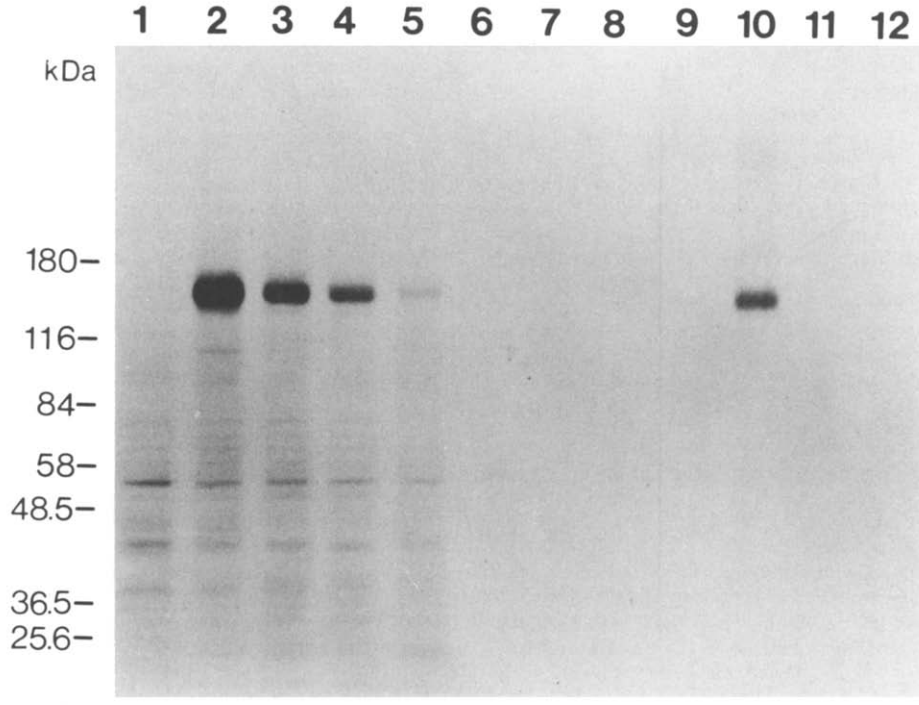

Fig. 1. [^{3}H]Azidopine photoaffinity-labeled membrane proteins from drug-sensitive and VBL-resistant cells. Membranes (80 μg protein) prepared from the J774.2 and J7.V1-1 cells were photolabeled in a final volume of 80 μl as described in Materials and Methods. Photolabeled membranes were analyzed by SDS–PAGE on 5–12% gradient gels. Lane 1: J774.2; Lanes 2–12: J7.V1-1. Lane 2: [^{3}H]azidopine labeling of membranes. Lanes 3–5: [^{3}H]azidopine labeling of membranes in the presence of a 250-, 500- and 1000-fold molar excess of nifedipine respectively. Lane 6: same as lane 2 except no UV irradiation. Lanes 7 and 8: [^{3}H]azidopine was irradiated for 10 min prior to the addition of membranes with no further irradiation (lane 7) or a 10-min irradiation after a 1-hr incubation at 25° (lane 8). Lanes 9–12: immunoprecipitation of [^{3}H]azidopine-labeled membranes from J7.V1-1. Photoaffinity labeling was carried out in the absence (lanes 9 and 10) or presence (lanes 11 and 12) of a 1000-fold molar excess of nifedipine. A polyclonal antibody specific for the MDRG (lanes 10 and 12) or pre-immune serum (lanes 9 and 11) was used.

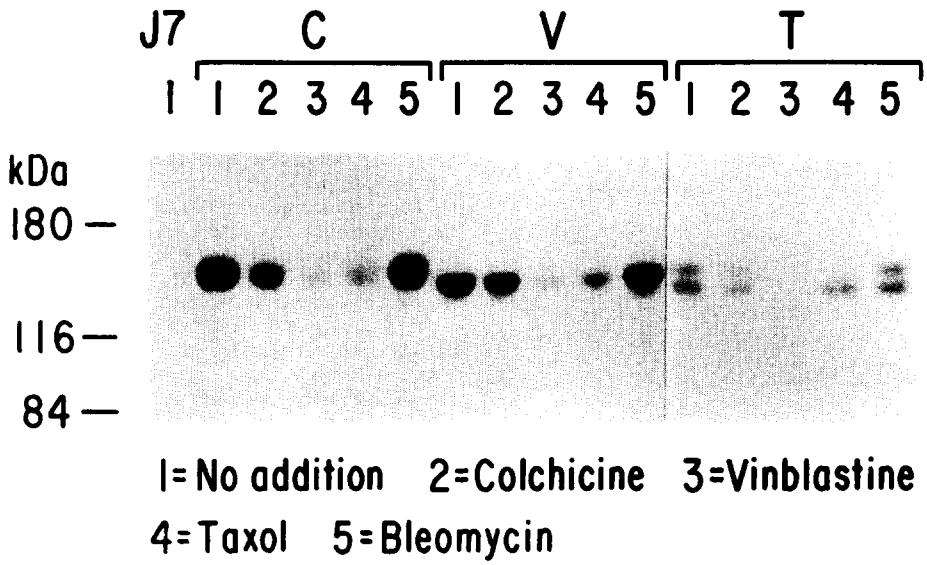

Fig. 2. Effects of VBL, CLC, TAX and bleomycin on photoaffinity labeling of MDRGs with [^{3}H]azidopine. Membranes were prepared from J774.2 (J7), J7.C1-100 (C), J7.V1-3 (V) and J7.T1-50 (T) cells. Membranes (68 μg protein) were photolabeled in a final volume of 68 μl. This mixture was preincubated for 1 hr at 25° in the absence of presence of a 1000-fold molar excess of CLC, VBL, TAX or bleomycin, irradiated, and analyzed as in Fig. 1. To quantitate the radioactivity in the MDRGs, the radiolabeled bands were excised from Coomassie-blue stained gels and soaked in 90% Beckman Tissue Solubilizer (BTS-450) overnight; radioactivity was determined by liquid scintillation counting.

J7.V1-1, 36% of the total radioactivity in the gel was present in the MDRG, which accounts for ~3% of the total protein in the membrane preparation. Calcium (0–10 μM) had no significant effect on labeling. The dissociation constant for [^{3}H]azidopine in reversible binding experiments with our crude microsomal membrane preparations from J7.V1-1 was ~63 nM. Membranes prepared from J7.T1-50 revealed a radioactive doublet after photolabeling with [^{3}H]azidopine (Fig. 2). There was essentially no labeling in the 130–150 kDa range in membranes from either J774.2 (Figs. 1 and 2) or revertant J7.C1-100 cells that were grown in the absence of CLC for 8 months (data not shown). Control experiments indicated that photolabeling was not due to the covalent binding of the photolyzed products of [^{3}H]azidopine with the MDRG, since the MDRG was not labeled if the drug was UV irradiated prior to incubation with membranes (Fig. 1). Since unlabeled azidopine was not available, the ability of nifedipine (also a 1,4-dihydropyridine calcium antagonist) to compete with azidopine binding was determined. In the presence of increasing concentrations (250-, 500-, 1000-fold molar excess) of nifedipine, radiolabeling of the MDRG was reduced by 42, 64 and 80%, respectively, in J7.V1-1 cells (Fig. 1). This inhibition was specific since the radiolabeling of other membrane proteins (the minor bands seen by fluorography) by [^{3}H]azidopine was not altered significantly by increasing concentrations of nifedipine. In some experiments, a minor radioactive band seen at 110 kDa could be displaced by nifedipine, although this result was not observed consistently. Recently, it was reported that membranes from MDR Chinese hamster lung cells are photolabeled with [^{3}H]azidopine and that this labeling is altered by calcium antagonists [20].

The effects of CLC, VBL and TAX, the drugs used for selecting our MDR cell lines, on [^{3}H]azidopine labeling were determined in each drug-resistant cell line (Fig. 2). A 1000-fold molar excess of VBL reduced the labeling of the MDRGs by 85% in each cell line, whereas CLC, at the same molar excess, reduced the labeling of the MDRGs by only 25–40%. TAX reduced labeling of the MDRG in J7.V1-1, J7.C1-100 and J7.T1-50 by 40–60%. In the latter cell line, the labeling of the upper band tended to be more reduced than that of the lower band. Bleomycin, an antitumor agent to which the three resistant cell lines demonstrated no cross-resistance [13], had a slight stimulatory effect on [^{3}H]azidopine photolabeling of the MDRGs. The results suggest a relationship between the binding site for azidopine and the binding site(s) for CLC, TAX and particularly VBL on the MDRG. With the evidence that is available, it is not possible to differentiate between a common binding site for azidopine and VBL or an allosteric effect of azidopine binding on VBL binding. The possibility that CLC, VBL, and TAX have individual binding sites on each of the MDRGs must be considered. It should be noted that each of the resistant cell lines is markedly more resistant against the drug against which it was selected [13] and that the MDRGs from the three resistant cell lines do not have identical electrophoretic mobilities [16].

The World Health Organization has subdivided the calcium antagonists into six types: verapamil-like (I), nifedipine-like (II), diltiazem-like (III), flunarizine-like (IV), prenylamine-like (V), and others (VI, such as perhexiline) [21]. The effects of these drugs on [^{3}H]azidopine photoaffinity labeling of the MDRGs were examined (Fig. 3). Types I, II, III, and IV had inhibitory effects on the labeling of the MDRGs in all three drug-resistant cell lines. Types V, VI and bepridil have stimulatory effects on [^{3}H]azidopine labeling of the MDRGs in J7.V1-1 and J7.Cl-100. In J7.T1-50, a stimulatory effect was apparent in the upper MDRG band but not in the lower band although the trend was similar. Bepridil at a 250- and 500-fold molar excess increased the labeling of the MDRG by 50 and 26%, respectively, in J7.V1-1. Immunoprecipitation of the [^{3}H]azidopine-labeled membranes from J7.V1-1 with an antibody specific for the MDRG [15] indicated that pre-

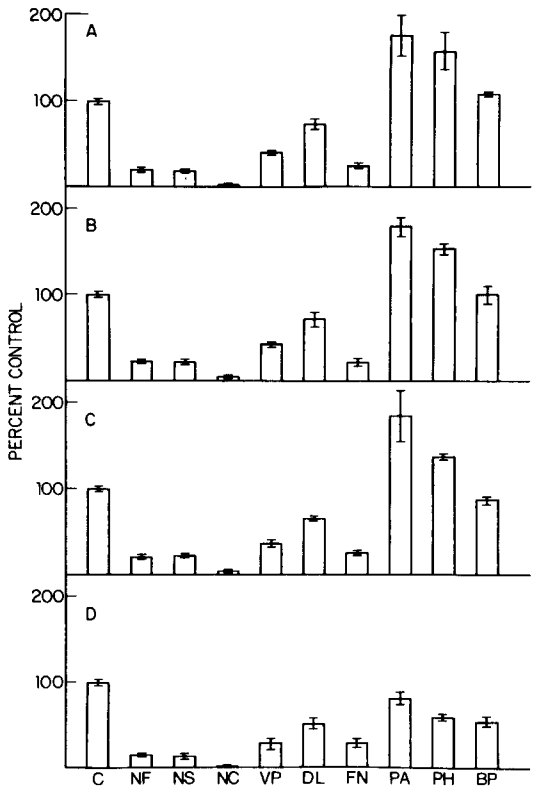

Fig. 3. Effects of calcium antagonists on the photoaffinity labeling of MDRGs with [^{3}H]azidopine. Membranes were prepared, incubated, and analyzed as in Fig. 2. Photoaffinity labeling was carried out in the absence or presence of a 1000-fold molar excess of various calcium antagonists. Abbreviations: C, control; NF, nifedipine; NS, nisoldipine; NC, nicardipine; VP, verapamil; DL, diltiazem; FN, flunarizine; PA, prenylamine; PH, perhexiline; and BP, bepridil. NF, NS and NC belong to the 1,4-dihydropyridine class. The MDRG bands were excised, and radioactivity was determined. Data is expressed as percent control (control = 20,970 dpm) with standard deviations (N = 3). A = J7.V1-1; B = J7.C1-100; C = J7.T1-50 (upper band); and D = J7.T1-50 (lower band).

nylamine, perhexiline and bepridil at a 500-fold molar excess increased the labeling of the MDRG by 137 ± 23, 80 ± 18 and 29 ± 7% (mean ± SD, N = 3) respectively. Similar results were obtained when radiolabeling was determined by excising the gel slice containing the MDRG and determining its radioactivity by liquid scintillation counting. This stimulatory effect caused by prenylamine, perhexiline and bepridil could result from an increase in reversible binding of azidopine to the MDRG or an increased covalent coupling of the drug. These classes of drugs may have a positive allosteric effect on [^{3}H]azidopine labeling by binding to a separate site on the MDRG. The observation that the stimulatory effect was much more pronounced in the upper than the lower MDRG band in J7.T1-50 suggests that the two MDRGs in J7.T1-50 can be distinguished in other ways besides their electrophoretic mobilities.

Although the effects of nicardipine and prenylamine on [^{3}H]azidopine photoaffinity labeling of MDRGs were opposite, both drugs enhanced the sensitivity of J7.V1-1 to VBL. The EC_{50} (the drug concentration that inhibits cell division by 50% after 72 hr) for VBL in J7.V1-1 was 1.4 μM. The addition of 2 μM nicardipine or prenylamine reduced the EC_{50} to 0.66 and 0.35 μM respectively (N = 3). It should be noted that the EC_{50} for nicardipine alone was >50 μM, whereas that for prenylamine was 6.3 μM in J7.V1-1.

The [^{3}H]azidopine-labeled MDRGs have been subjected to tryptic digestion, and the resulting peptides have been resolved by reversed phase HPLC. The elution profile (Fig. 4) revealed a major radioactive peak with a retention time of 61.5 min. The intensity of a less hydrophobic peak with a retention time of 57 min varied between experiments. The position of the major peak was essentially identical for all the MDRGs studies (VBL, CLC and the TAX upper and lower bands). These results strongly suggest that there is one major binding site for azidopine on the MDRG molecule.

Although the relationship, if any, between the MDRG and the voltage-dependent calcium channel is not clear, our experiments indicate that azidopine binds to the MDRG and that this binding is influenced by the presence of other calcium antagonists and by VBL, CLC, and TAX. A dissociation constant for azidopine of 0.35 nM has been reported in skeletal muscle transverse tubule membranes, a rich source for dihydropyridine receptors [22]. This value suggests that azidopine has a higher affinity for the calcium channel than for the MDRG. The ability to radiolabel the MDRG with azidopine provides an important new tool for further characterization of the structure and function of the

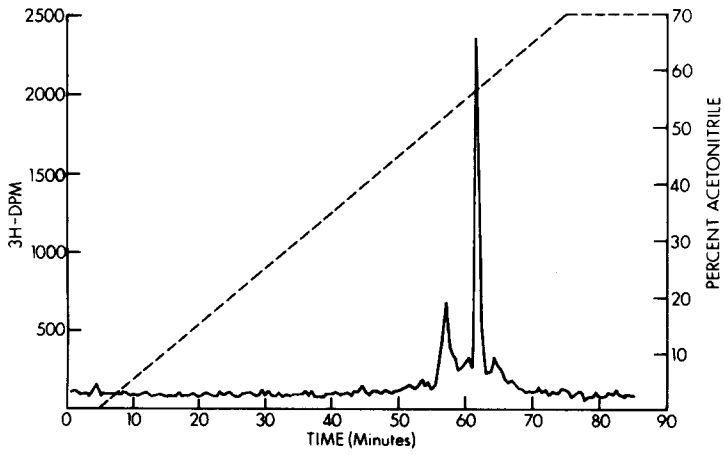

Fig. 4. Analysis of radiolabeled peptides by HPLC after trypsin digestion of the [^{3}H]azidopine-labeled MDRG. The radiolabeled MDRG band prepared from J7.V1-3 cells was excised from gels, washed, and digested by trypsin, and the digested peptides were analyzed by HPLC as described in Materials and Methods.

MDRG. A role for calcium antagonists in reversing drug resistance deserves extensive exploration.

In summary, a photoactive calcium antagonist, [^{3}H]azidopine, can specifically radiolabel the MDRG. Other calcium antagonists and drugs used to select our resistant cells influenced [^{3}H]azidopine labeling of the MDRG. Analysis of tryptic peptides from the [^{3}H]azidopine-labeled MDRG suggested that this calcium antagonist has a specific binding site on the MDRG.

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Ascorbic acid-induced binding of [^{125}I]-iodocyanopindolol to non-beta-adrenoceptor sites in guinea-pig trachea

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[^{125}I]-Iodocyanopindolol (I-CYP) is a potent, selective radioligand for beta-adrenoceptors [1–4]. However, recent evidence indicates that I-CYP also binds with high affinity to a population of non-beta-adrenoceptor sites in dog kidney [5]. Furthermore, we have recently shown that in the presence of ascorbic acid, I-CYP binds to non-beta-adrenoceptor sites in guinea-pig trachea [6]. The present study further examines the characteristics of ascorbic acid-induced I-CYP binding in airway tissue.

Methods

Tissue preparation, I-CYP binding and autoradiography. Male guinea-pigs (SR/C Tricolour; 400–450 g) and male Wistar rats (220–250 g) were stunned and exsanguinated and the trachea removed. Bronchi (2–3 mm i.d.) were also dissected from lung from pigs freshly slaughtered at a local abattoir. Airway tube segments were submerged in 6% dextran 70, containing 5% dextrose (Macrodex, Pharmacia) and rapidly frozen in isopentane cooled in liquid nitrogen. Each frozen block contained airway tissue from at least 3 separate animals. Serial, transverse, 10-μm sections were cut at −30° and mounted and thawed onto washed gelatinized glass slides. Binding and autoradiographic experiments using I-CYP (5–320 pM; Amersham), were conducted as previously described [6]. In some experiments, tissue sections were exposed to I-CYP (50 pM) for 2 hr in the presence of ascorbic acid, dithiothreitol, L-cysteine (Sigma Chemical Co.) or sodium metabisulphite (BDH) (1 nM–1 mM). Unless otherwise stated, propranolol (10 μM) (ICI) was routinely included to abolish I-CYP binding to beta-adrenoceptors. Various cations as well as drugs from several pharmacological groups were also tested for their effects on I-CYP binding to non-beta-adrenoceptor sites in the presence of ascorbic acid.

Results and discussion

Specific I-CYP binding in guinea-pig and rat trachea and in pig bronchus was saturable, involving high affinity sites (dissociation constant K_d = 69 ± 9; 80 ± 14 and 66 ± 12 pM respectively). Specific binding was reduced to background levels in the presence of propranolol (10 μM) or (−)-isoprenaline (200 μM), indicating reversible binding was to