A Novel Electron Paramagnetic Resonance Approach to Determine the Mechanism of Drug Transport by P-glycoprotein*

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ATP-driven pumping of a variety of drugs out of cells by the human P-glycoprotein poses a serious problem to medical therapy. High level heterologous expression of human P-glycoprotein, in the yeast Saccharomyces cerevisiae, has facilitated biophysical studies in purified proteoliposome preparations. Membrane permeability of transported drugs and consequent lack of an experimentally defined drug position have made resolution of the transport mechanism difficult by classical techniques. To overcome these obstacles we devised a novel EPR spin-labeled verapamil for use as a transport substrate. Spin-labeled verapamil was an excellent transport substrate with apparent turnover number, $K_m$ and $K_i$ values of 5.8 s\(^{-1}\), 4 $\mu$M, and 210 $\mu$M, respectively, at pH 7.4 and 37 °C. The apparent affinities were $10^4$-fold higher than unlabeled verapamil. Spin-labeled verapamil stimulated ATPase activity $5^\circ$-fold, was relatively hydrophobic, and had a very low flip-flop rate, making it an ideal transport substrate. The $K_m$ for MgATP activation of transport was 0.8 $\mu$M. By measuring the mobility of spin-labeled verapamil during transport experiments, we were able to resolve the location of the drug in proteoliposomes of suspended steady state gradients of spin-labeled verapamil within the range of $K/K_m$ ratios were observed.

The resistance of cancer cells to a wide variety of anti-cancer drugs leads to failure of chemotherapy and remains a major medical problem. P-glycoprotein (Pgp)\(^1\) mediates such multidrug resistance through its ability to transport various hydrophobic compounds across the plasma membrane (1). This feature of Pgp is of great clinical interest.

P-glycoprotein belongs to the ATP-binding cassette transporter family (2). This protein consists of two homologous domains; each domain has six transmembrane helices and an ATP-binding site. Photolabeling and genetic studies have indicated that drug-binding site(s) are located in the transmembrane region (reviewed in Ref. 3). Binding of drugs stimulates ATP hydrolysis at ATP-binding sites, which is coupled to drug transport (4). Transport substrates of Pgp are generally hydrophobic compounds that partition into the bilayer. Many of them are positively charged or uncharged. Studies utilizing fluorescent substrates have shown that Pgp removes drugs from the cytoplasmic leaflet of plasma membranes and exports them to the external aqueous medium (5–8). This is known as the “hydrophobic vacuum cleaner model” (1). To understand the mechanism of drug transport and energy coupling, the location of drug must be determined without ambiguity. Due to high lipid partition coefficients, high diffusion rates, as well as nonspecific drug binding in conventional assays, it is difficult to investigate the molecular details of substrate binding and transport by Pgp. In consequence, the mechanism of drug transport coupled to ATP hydrolysis is still not resolved.

Binding of an amphipathic molecule to liposomes changes its mobility and accessibility. EPR spectroscopy is a good tool to monitor quantitatively changes in the mobility and accessibility (9, 10). Here we report a new strategy to investigate the transport mechanism of P-glycoprotein using a new spin-labeled transport substrate.

MATERIALS AND METHODS

Yeast Strain and Media—Yeast strain BJ5457/EpMDR1HIS that expresses $10^4$-His-tagged human P-glycoprotein (MDRI gene product; see Ref. 11) was cultured at 30 °C in 36 liters of Synthetic Dextrose (SD) media supplemented with 10% (v/v) glycerol to improve Pgp expression. Additional $10^6$ concentrated SD media were added at 1.0 and 2.2 OD\(_{600}\) units. Cells were harvested at 3.0 OD\(_{600}\) units and frozen by liquid nitrogen.

Preparation of Microsomes and Proteoliposomes—Frozen cells were thawed in deionized water containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and precipitated by centrifugation at 10,000 × g. Cells (~130 g) were resuspended in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3 M sucrose, 5 mg/ml bovine serum albumin, 25 mM β-mercaptoethanol, 1 mM PMSF, 2 mM pepstatin, 1 mM leupeptin, 1 mM benzamide, 1 mM diisopropyl fluorophosphate). Cells were broken and homogenized on ice using silica-zirconia beads in a Bead-Beater (Biospec Products Inc.). Three 4-min cycles were employed, and fresh 1 mM diisopropyl fluorophosphate was added at each cycle. Beads were washed by fresh homogenization buffer and combined with cell lysates. Crude microsomes were prepared, and P-glycoprotein was purified as described previously (11).

P-glycoprotein containing proteoliposomes were made by dialysis as described previously (12) utilizing 0.1% (w/v) “mixed lipid” composed of 60% (w/v) Escherichia coli ether/acetone-precipitated lipid, 17.5% egg phosphatidylcholine, 10% bovine brain phosphatidylserine, and 12.5% cholesterol (lipids were from Avanti polar lipids). Reconstituted vesicles were suspended in EPR buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, and 50 mM KCl) plus 0.1 mM dithiothreitol and centrifuged again at 270,000 × g for 60 min. Pellets were resuspended in a small amount of the same buffer. Control liposomes, lacking Pgp, were prepared in parallel. An equivalent lipid/detergent mixture (25 mM Tris-HCl, pH 7.5, 250 mM imidazole HCl, 2 mM MgSO\(_4\), 50 mM Na\(_2\)SO\(_4\), 2 mM ATP, 20% (v/v) glycerol, 1.4% (w/v) octyl glucoside, 0.1% (w/v) mixed lipid, 2 mM β-mercaptoethanol, 1 mM PMSF) was passed through a washed nickel-nitrilotriacetic acid column (Qiagen). Eluates were dialyzed and...
control liposomes collected by centrifugation. Lipid concentrations were measured by the method of Andree and Soedjak (13). Other routine methods can be found in Figler et al. (11).

**Synthesis of Spin-labeled Verapamil**—Spin-labeled verapamil (SL-verapamil) was synthesized from verapamilthyletheneisothioulibromide (MTS-verapamil; Toronto Research Chemicals) and proxylmaleimide (Aldrich). All procedures were carried out in the dark at room temperature. For chemical reduction, 800 μl of 0.65 mM MTS-verapamil in 50 mM HEPES/NaOH, pH 8.5, and 70% (v/v) methanol was applied to an immobilized dithiothreitol gel column (0.8 g of Reduc-tacryl, Calbiochem) pre-equilibrated with the same buffer. After 1 h, reduced MTS-verapamil was recovered by washing the column with 6 ml of the same buffer and was then coupled to 5 μmol of proxylmaleimide during a 1-h incubation. Crude SL-verapamil was concentrated to ~400 μl by an argon stream and then purified by high performance liquid chromatography using an XTerra RP column (4.6 × 150 mm, Waters) run with 60% (v/v) methanol and 4 mM Tricine/NaOH, pH 8.5, as the mobile phase. Elution of unrelated species and products were monitored at 278 nm. Methanol and water were evaporated from the SL-verapamil fraction by an argon stream. The resultant solution was mixed with an equal volume of dimethyl sulfoxide and stored under argon at ~80 °C. A further high performance liquid chromatography run checked the purity of SL-verapamil, and the correct molecular mass of 753 Da was confirmed by mass spectrometry.

**ATPase Assays**—Two different ATPase assays were employed. For conventional assay, activity was measured at 37 °C in an ATPase mixture containing 20 mM Tris-Cl, pH 7.4, 10 mM ATP, 15 mM MgSO4 (11). In the other assay, the reaction was started by addition of various concentrations of MgATP to 35 μl of EPR buffer containing 2 μM P-glycoprotein, 1 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, and 0.5 mM MgSO4. Temperature was strictly controlled at 23 °C. Samples (5 μl) were withdrawn at appropriate times to 100 μl of ice-cold 8 mM EDTA (pH 8) to stop further reaction. Determination of liberated inorganic phosphate was as described previously (14).

Drug titrations of ATPase activities were fitted to an activity-partitioning model in which basal and drug-stimulated activities are co-dependent. Thus Pgp partitions between two forms, an uncoupled form in the presence of activating drug. At maximal activity all Pgp exists in the coupled form. Both activities are inhibited by inhibitory concentrations of drug. The steady state velocity equation is given by Equation 1,

\[
\frac{v}{v_{0}} = \left[1 - \frac{K_{d} + [\text{drug}]}{K_{a} + [\text{drug}] + [\text{drug}]}\right] \left(1 - \frac{K_{p} + [\text{lipid}]}{K_{p} + [\text{lipid}]}\right)
\]

(Eq. 1)

where \(v\) is the ATPase activity; [drug] is the drug concentration; \(B\) is the basal ATPase activity; \(D\) is the maximal ATPase activity associated with drug activation; \(K_{a}\) is the Michaelis constant for drug activation, and \(K_{p}\) is the inhibition constant for drug inhibition.

**EPR Measurements**—Continuous wave EPR spectra were taken at 23 °C using a Bruker EMX X-band EPR spectrometer fitted with a loop gap resonator (Medical Systems). Samples were loaded in TFX capillaries (IGC Medical Advances Inc.). For measurement of partition coefficients, EPR spectra of SL-verapamil were recorded with various concentrations of lipid in the EPR buffer. The molar partition coefficient, \(K_{p}\), is a drug partition coefficient that has the units of m^−1 with respect to lipid (organic phase) concentration in the total mixture. For applications involving liposomes and proteoliposomes, this constant is useful in calculating the amounts of free and partitioned drug for any concentration of known lipid because the volume ratio of organic to aqueous phases is subsumed in the concentration term. \(K_{p}\) was calculated according to Victor and Caffo (10) as shown in Equations 2 and 3.

\[
\text{fraction of probe partitioned into lipid} = \frac{P_{\text{lipid}}}{P_{\text{total}} + P_{\text{lipid}}} (\text{Eq. 2})
\]

and

\[
\text{fraction of probe partitioned into lipid} = \frac{K_{p}[\text{lipid}]}{1 + K_{p}[\text{lipid}]} (\text{Eq. 3})
\]

Here, \(P_{\text{total}}\) and \(P_{\text{lipid}}\) are peak to peak intensities of the high field signal (\(m_{r} = −1\) resonance) with lipid and without lipid, respectively. The fraction of probe partitioned into lipid at any particular experimental lipid concentration was calculated by Equation 2 (see Fig. 4, insert).

Due to nonspecific binding of SL-verapamil to plastics in the absence of lipids, the peak to peak intensity decreased significantly without lipid. Extrapolation of lipid titration curves to zero lipid concentration was used to calculate the corrected \(P_{\text{lipid}}\). \(K_{p}\) was obtained by fitting Equation 3 to the lipid-partitioned probe data as a function of lipid concentration (see Fig. 4). As the lipid/phase rate of SL-verapamil was very slow ("Results"), the lipid concentration ([lipid]) refers to the external lipid leaflet concentration only.

For transport assays, 10 mM MgATP or various given MgATP concentrations were added to 5 μl of reaction mixture containing 0.4–2 μM reconstituted Pgp, 50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 50 mM KCl, 5 mM dithiothreitol, 0.1 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, 0.5 mM MgSO4, and 10–300 μM SL-verapamil and mixed by tituration. Monitoring of high field EPR spectra was started immediately. Each scan (10 G sweep width) was recorded for analysis. A wire type thermocouple probe was used to measure temperature of the resonator. Apparent transport rate constants were obtained by exponential fitting of the data.

**Measurements of Drug Partitioning into Olive Oil**—Drug partitioning into olive oil (Fluka) was measured according to Zamora et al. (15). Drugs were solubilized in buffer containing 100 mM NaHPO4/ NaH2PO4, pH 7.5, and added to an equal volume of olive oil and mixed vigorously for 30 min at 23 °C. Mixtures were separated by centrifugation. Concentrations were determined by EPR spectra or by optical absorbance of the aqueous phase in the case of unlabeled verapamil.

**Measurement of Vesicle Size**—Vesicles were diluted to 100 μg/ml with EPR buffer, and vesicle diameter was measured by dynamic light scattering on a DynaPro-MS800 (Protein Solutions). Vesicle hydrodynamic radii were calculated using Dynamics software (Protein Solutions).

**Calculation of Spin-labeled Verapamil Concentration in Each Compartment**—Concentrations of spin probe in each compartment were calculated utilizing measurements of amounts of free and lipid-partitioned SL-verapamil, concentrations of SL-verapamil transported, vesicle hydrodynamic radii, lipid concentrations, and partition coefficients (\(K_{p}\)). From these measurements the additional parameters of lipid leaflet volumes, compartmental areas, and lipid concentrations were calculated assuming an average weighted molecular mass of 583 Da for the mixed lipid preparations and a bilayer thickness of 4 nm (16). Because the external aqueous volume of the medium was much larger than the aqueous lumen volume, the signal from free aequous SL-verapamil was initially equated with the external aqueous concentration (a very good approximation; see "Results"). Employing the value determined above, together with the measured outer leaflet lipid concentration, the fraction of spin probe partitioned into the outer leaflet was calculated using Equation 3. The fraction partitioned was then converted into concentration units. Similarly, employing the amount of transported SL-verapamil, at any given time point, together with the calculated inner leaflet lipid concentration, the concentration of luminal free and inner leaflet-partitioned spin probe were calculated using Equation 3. The majority of the transported SL-verapamil was lipid-partitioned ("Results"). Initially calculated concentrations were then slightly readjusted to account for the small luminal aqueous concentrations measured.

**RESULTS**

**ATPase Activity**—Spin-labeled verapamil was synthesized by reduction of MTS-verapamil followed by modification with proxyl-maleimide (see "Materials and Methods"). The molecular size of SL-verapamil (753 Da) is substantially greater than that of verapamil (455 Da). Additionally SL-verapamil carries a permanent positive charge (Fig. 1). These features could have affected the ability of the P-glycoprotein to recognize SL-verapamil as a substrate. We measured activation of Pgp ATPase activity as a preliminary test of substrate recognition (12, 17). Fig. 2 shows that SL-verapamil activated Pgp ATPase activity by almost 5-fold, similar to verapamil. Apparent \(K_{a}\) and \(K_{p}\) values were 4.3 and 206 μM, respectively. These values were significantly lower than those of verapamil (62 and 640 μM, respectively) indicating spin-labeled verapamil is a higher affinity substrate than verapamil.

**Partitioning of Spin-labeled Verapamil into Liposomes**—In the hydrophobic vacuum cleaner model of drug transport (1), Pgp takes up substrates from the cytoplasmic leaflet of the
plasma membrane and transports them to the aqueous medium surrounding the cell. Thus partitioning of drugs into the lipid bilayer is a major determinant of the apparent $K_m$ of drug transport (18, 19). We measured the SL-verapamil partitioning into liposomes by monitoring mobility changes using EPR spectroscopy. The aqueous solution spectra of SL-verapamil (Fig. 3 A) and proxyl-maleimide (not shown) were similar with three sharp peaks (resonance lines) characteristic of high mobility at the nitroxide moiety. However, when SL-verapamil was mixed with liposomes (121 mM lipid), a low mobility signal (broadened peaks) appeared (Fig. 3 B), and the intensity of the high mobility signal (sharp peaks) was decreased. In contrast, the proxyl-maleimide signal did not change with the addition of liposomes (not shown). Similarly, in the case of spin-labeled amphipathic basic peptides, partitioning of the peptides into liposomes lowered the mobility (10). Thus, it is reasonable to assign the low and high mobility portions of the signals to liposome-partitioned and aqueous phase SL-verapamil, respectively.

Because the high mobility signal at high field ($m_1$ resonance) is well separated from the low mobility signal (compare Fig. 3, A and B), the peak to peak intensity at high field (PPI) can be used to calculate the aqueous phase concentration of free spin probe. The molar partition coefficient of SL-verapamil into mixed lipids ($K_p$) was determined by liposome titrations using a fixed concentration of 50 $\mu$M SL-verapamil (Fig. 4; see "Materials and Methods"). The determined value of $K_p$ was unchanged when 25 $\mu$M SL-verapamil was used in liposome titrations (not shown). Thus under normal experimental conditions (10 mM lipid), it is expected that most of SL-verapamil will be located in the aqueous phase as was seen (Fig. 3 C). The partition coefficient ($P_v$) of SL-verapamil into olive oil was found to be only 0.35 in contrast to a value of 42 for verapamil. Thus SL-verapamil appears to be 120-fold more hydrophilic than verapamil. We think that this hydrophilicity results primarily from the fixed positive charge of this compound.

**Spin-labeled Verapamil Transport by P-glycoprotein Vesi-
cles—**Verapamil has long been known to be a good transport substrate for P-glycoprotein (20). Under our experimental conditions, the expected small low mobility signal of SL-verapamil bound directly to Pgp could not be resolved from the large low mobility signal of SL-verapamil partitioned into liposomes. However, in the presence of Pgp and MgATP, there was a time-dependent drop of the high mobility signal with a concomitant increase of the low mobility signal (compare Fig. 3, C and D). Such signal changes were not observed on addition of non-hydrolyzable MgAMPPNP (Fig. 5) or MgATP plus orthovanada-

tate (not shown). Furthermore, MgATP did not alter the EPR spectrum of SL-verapamil with liposomes in the absence of Pgp (not shown). The decrease of high mobility signal indicates a decrease of free spin probe in the aqueous phase. This decrease
concentration in the aqueous phase. The ratio of lipid-partitioned fraction of SL-verapamil was then calculated as described under "Materials and Methods" using Equation 2. Molar partition coefficient ($K_p$) was obtained from the fit of the data to Equation 3 (dashed line; see "Materials and Methods" for further details).

FIG. 4. Lipid partitioning of spin-labeled verapamil. EPR spectra were measured with 50 μM SL-verapamil in EPR buffer, at 23°C and pH 7.5, containing the indicated amount of lipid in the outer leaflet of added mixed lipid liposomes. Inset shows the primary data; PPI values for the high field resonance ($m_I = -1$) are plotted against lipid concentration of outer leaflet. From this plot the corrected aqueous signal ($PPI_{corr}$) in the absence of lipids is calculated by back extrapolation. The lipid-partitioned fraction of SL-verapamil is then calculated as described under "Materials and Methods" using Equation 2. Main graph shows the calculated lipid-partitioned fraction of SL-verapamil plotted against lipid concentration of outer leaflet. Molar partition coefficient ($K_p$) was obtained from the fit of the data to Equation 3 (dashed line; see "Materials and Methods" for further details).

FIG. 5. Spin-labeled verapamil transport by P-glycoprotein vesicles. Reactions were started by addition of 10 mM MgATP to EPR buffer containing 25 μM SL-verapamil, 2 μM P-glycoprotein, and an ATP regeneration system at 23°C and pH 7.5. Recording of EPR spectra was initiated immediately. Peak to peak intensity (PPI) of high field signal ($m_I = -1$ resonance) was converted to the concentration of free spin probe in the aqueous phase. The ratio of PPI values to free probe concentration in μM units was ~150. See "Materials and Methods" for further details. △, 10 mM control MgAMPPNP added; ○, 10 mM MgATP added; ■, 1 mM vanadate was added at 20 min after MgATP addition; □, 20 mM EDTA was added at 20 min after MgATP addition.

by MgATP corresponded to a decrease of ~10 μM free SL-verapamil. This value was ~5-fold larger than the Pgp concentration of 2 μM in Fig. 5 and up to 50-fold larger in other experiments (not shown). These results indicate that this new spin probe was transported by P-glycoprotein.

Spin-labeled verapamil transport was confirmed directly by separation of vesicles from the suspending medium by centrifugation after MgATP addition (Fig. 6). Double integration of EPR spectra indicated 45% of spin probe was recovered from the vesicle fraction. The majority of SL-verapamil signal in the separated vesicles was of low mobility type, indicating that the lipid-partitioned form was the predominant species in vesicles (Fig. 6, spectra). In contrast, the supernatant after centrifugation had only the high mobility type signal, which represented 55% of total spin probe present (Fig. 6). The signals were additive, thus total spin probe was recovered in the two fractions. Furthermore, calculated amounts of SL-verapamil in the different compartments were similar when estimated from the results prior to and after separation by centrifugation. Overall the results indicate that SL-verapamil is transported into vesicles and is then partitioned into lipid, probably to the inner leaflet of proteoliposomes. Because the intravesicular aqueous space is so limited (Fig. 7), the effective lipid concentration inside proteoliposomes is very high. Thus, most of SL-verapamil inside of vesicles is expected to be lipid-partitioned after transport to the lumen. Based on the $K_p$ value determined (Fig. 4) and calculation of internal lipid concentrations, it is expected that 60–70% of SL-verapamil would be partitioned into the lipid phase.

Addition of vanadate or EDTA to inhibit Pgp activity did not induce release of accumulated SL-verapamil for up to 3 h (e.g., Fig. 5), whereas the addition of the detergent dodecyl-β-D-maltopyranoside allowed SL-verapamil re-equilibration with the medium (not shown). Thus the vesicles into which SL-verapamil accumulated were tightly sealed. The rate of partitioning of SL-verapamil into lipid was very fast and could not
be resolved experimentally. Because the association of SL-verapamil with lipids is a partitioning phenomenon, it is expected that the release rate of SL-verapamil from lipids would also be very fast. This was confirmed by experiments in which lipid vesicles were preincubated with high concentrations of SL-verapamil, which rapidly partitioned into the outer leaflet. Upon dilution of these vesicles there was a rapid re-equilibration of SL-verapamil with the aqueous phase (results not shown). Thus the results of Fig. 5 suggest that the flip-flop rate of transported SL-verapamil between the two leaflets of the membrane was negligible over the assay time course. Similarly, the rate of re-equilibration of transported SL-verapamil from the centrifugation-separated and resuspended vesicles (Fig. 6) was very slow, confirming that the flip-flop rate was slow.

Apparent $K_m$ and $V_{max}$ values for SL-verapamil transport of 13.5 and 216 $\mu$M, respectively, were obtained from initial rates of transport as a function of added SL-verapamil concentration (not shown). These values were similar to the analogous constants determined in ATPase assays (Fig. 2).

**Gradient Formation by P-glycoprotein**—To calculate the concentration of SL-verapamil in each compartment of the proteoliposome suspension, the average vesicular radius was determined by dynamic light scattering. The averaged vesicle hydrodynamic radius was 42 nm indicating that typical unilamellar vesicles were present. Concentrations of SL-verapamil in both aqueous phases and in the outer and inner leaflets were calculated employing the experimental results of amounts of free and lipid-partitioned SL-verapamil, and concentrations moved together with knowledge of vesicle radii, partition coefficients, and lipid concentrations. During a transport experiment, the decreased amount of free spin probe was taken to represent the amount of transported SL-verapamil. This was verified by the centrifugation experiment (Fig. 6). Free spin probe concentration in the lumen was calculated using the molar partition coefficient and inner leaflet lipid concentration (see “Materials and Methods” for further details). Calculated results for the experiment shown in Fig. 6 are illustrated schematically in Fig. 7. After maximum transport, the luminal SL-verapamil concentration was 289 $\mu$M. There was a 12.6-fold gradient of SL-verapamil between both aqueous phases across the membrane. In addition, there was a 10.6-fold concentration gradient between the outer and inner leaflets of the vesicle. In another series of transport experiments the concentration of SL-verapamil was varied from 10 to 380 $\mu$M. It was found that the final luminal aqueous phase concentration increased with increasing external aqueous SL-verapamil, and the aqueous phase gradient varied in the range 7–25-fold (data not shown). For reasons discussed later, these gradients are underestimates of the true gradients and represent minimal estimates.

**DISCUSSION**

The hydrophobic vacuum cleaner model of drug transport by P-glycoprotein is supported by various studies (see Introduction). To examine and test this model, it is imperative to know the drug location during the transport process. For example, the actual concentration of drugs in the cytoplasmic leaflet directly affects enzyme activity because P-glycoprotein takes up its transport substrates from this leaflet. However, most of the transport substrates are highly hydrophobic and highly permeable to membranes. This feature of P-glycoprotein substrates causes numerous and insurmountable technical problems for mechanistic transport studies by conventional meth-
ods (reviewed in Ref. 21). EPR methods using spin-labeled transport substrates can overcome many of these problems and offer the following advantages. First, the location of spin probe is easily identified by mobility and accessibility tests without sample separation. Second, EPR techniques are highly quantitative and insensitive to calibration artifacts. Third, interpretation of EPR signals is relatively straightforward, and there are few associated interference problems. This is in stark contrast to the situation where the transport substrates used are fluorescence probes (e.g. Refs. 5, 7, and 22), which are subject to many measurement artifacts including light-scattering, inner-filter effects, photobleaching, and multiple mechanisms of fluorescence quenching. Indeed, our new spin probe solved many previous technical problems and worked well in mechanistic studies of P-glycoprotein.

Spin-labeled verapamil has several unique features. It is more hydrophilic than verapamil. The partition coefficient of SL-verapamil into olive oil was 120-fold smaller than that of verapamil. The fixed positive charge of the spin-labeled verapamil may have increased its hydrophilicity (Fig. 1). Even with this higher hydrophilicity SL-verapamil had apparent $K_m$ and $K_I$ values that were up to 10-fold lower than the corresponding values for verapamil. Thus SL-verapamil appears to exhibit high specific binding to P-glycoprotein. Additionally, SL-verapamil stimulated ATPase activity about 5-fold (Fig. 2) and was transported by P-glycoprotein (Fig. 5). Attachment of the nitroxide spin probe to verapamil resulted in a better transport substrate. The high hydrophilicity of SL-verapamil facilitates transport measurements.

Transport studies were further aided by the very slow flip-flop rate of SL-verapamil in the bilayer ("Results") in contrast to the relatively high flip-flop rate of verapamil (23). We believe that if the fixed positive charge of SL-verapamil interacts with the negative surface dipole potential of the bilayer and also inhibits flip-flop activity across the thermodynamic barrier of the hydrocarbon core. For comparison, the translocation rate of TPP$^+$ across membranes was only 0.001–0.01 s$^{-1}$ (9). As SL-verapamil has a similar molecular topology and a larger size than TPP$^+$, the flip-flop rate of our probe would be expected to be very slow. Prior to transport, the majority of SL-verapamil was located in the bulk water phase outside of vesicles yielding characteristic high mobility EPR signals (Figs. 3C and 6). Once transported inside of the vesicles, most SL-verapamil was partitioned into the lipid bilayer, due to high internal lipid concentrations, yielding characteristic low mobility EPR signals (Figs. 3D and 6). Thus, on addition of ATP, the concentration of free SL-verapamil decreased outside, and the concentration of lipid-partitioned SL-verapamil increased inside. Both parameters could be easily monitored and analyzed as shown in this study.

Qualitative drug transport studies have long employed fluorescent transport substrates (e.g. Refs. 8 and 24). Ling and colleagues (5, 7, 22) employed fluorescent transport substrates where fluorescence intensities were high in the lipid phase and quenched in the aqueous phase precluding direct estimates of aqueous phase concentrations. In the latter studies, Pgp-mediated transport decreased total fluorescence in vesicular preparations, which was taken to indicate that drugs were transported into the luminal aqueous phase from the lipid phase. For this conclusion to hold, transport rates must be much higher than the drug-rebinding rate to the bilayer. Turnover was thus assumed to be limited by lipid drug-binding rates in apparent conflict with their own experimental observations (5).

However, alternative mechanisms of fluorescence quenching are also possible, e.g. self-quenching in inner leaflet at the high concentrations achieved (5, 25) leading to different interpretations of the results. In contrast, we found that the binding rate of SL-verapamil to lipid was fast, and the transport rates were relatively slow and rate-limiting (see under "Results"). As a consequence, on transport, SL-verapamil rapidly partitioned into the inner leaflet of the bilayer in our case. Another great advantage of our transport studies is that they can be performed with pure Pgp in defined proteoliposomes as opposed to crude plasma membrane vesicle preparations required for transport studies using membrane fluorescent probes (5).

For the results of Fig. 6, SL-verapamil concentration in the outer leaflet was 250 $\mu$M in equilibrium with 23 $\mu$M free external probe (Fig. 7). Interestingly, net transport ceased after 10–20 min even though there was sufficient external substrate in the aqueous phase. This was not due to steady state equilibrium between transport and leakage, as we did not detect SL-verapamil leakage from vesicles after addition of EDTA or vanadate (Fig. 5). Also the mobility of lipid-partitioned spin probe at the steady state was not significantly changed, and vesicles were still intact. Thus transport was not inhibited by increased membrane fluidity (26). Another unlikely possibility for net transport termination was that the concentration of SL-verapamil reached the maximum binding capacity of the membrane. In the case of Fig. 6, SL-verapamil in the inner lipid layer was only 2.7 $\mu$M (values up to 29 $\mu$M were observed when more SL-verapamil was used). The lipid to spin probe ratio of 425 was also significantly smaller than the maximum binding capacity for TPP$^+$ (9). The most likely possibility, which was also supported by kinetic simulations, is that the high concentration of luminal SL-verapamil directly inhibited Pgp activity by inhibiting drug unloading from the low affinity site. Experimentally determined luminal concentrations of SL-verapamil were in a range similar to determined $K_m$ values (see "Results"). If the low affinity drug-binding site faces the lumen (27), then the luminal drug concentration may correlate with the $K_m$ value for this site. Furthermore, the ratio of $K_i$ to $K_m$ would correlate with the maximum potential gradient formation by Pgp. In our case, the ratio of $K_i$ to $K_m$ for SL-verapamil was 16 under transport conditions. In good agreement with this, P-glycoprotein generated a 13-fold gradient (range 7–25-fold in other experiments) between the luminal and outside aqueous phases and an 11-fold gradient between the lipid leaflets (Fig. 7). Our calculations only generate minimal estimates of the gradient, which is expected to be larger, because the calculations were based on the assumption that all vesicles were tightly sealed (no open sheets) and were uniformly spherical.

Our new EPR approach resolved the technical problems of drug transport studies and is useful for understanding the molecular mechanism of drug transport and energy coupling by P-glycoprotein.

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