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Effects of the Membrane Dipole Potential on the Interaction of Saquinavir with Phospholipid Membranes and Plasma Membrane Receptors of Caco-2 Cells*

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The combined use of the membrane surface potential fluorescent sensor fluorescein phosphatidylethanolamine (FPE) and the membrane dipole potential fluorescent sensor di-8-ANEPPS to characterize the interaction of molecules with model and cellular membranes and to asses the influence of the dipole potential on the interaction is reported. The study of the human immunodeficiency virus protease inhibitor saquinavir with Caco-2 cells and phospholipid membranes reveals that the compound interacts with the lipidic bilayer of model membranes with a simple hyperbolic binding profile but with Caco-2 cells in a cooperative way involving membrane receptors. Additional studies indicated that colchicine acts as a competitor ligand to saquinavir and suggests, in agreement with other reports, that the identity of the saquinavir "receptor" could be P-glycoprotein or the multiple drug resistance-associated protein. The modification of the magnitude of the membrane dipole potential using compounds such as cholesterol, phloretin, and 6-ketocholestanol influences the binding capacity of saquinavir. Furthermore, removal of cholesterol from the cell membrane using methyl-β-cyclodextrin significantly decreases the binding capacity of saquinavir. Because removal of cholesterol from the cell membrane has been reported to disrupt membrane domains known as "rafts," our observations imply that the membrane dipole potential plays an important role as a modulator of molecule-membrane interactions in these membrane structures. Such a role is suggested to contribute to the altered behavior of receptor-mediated signaling systems in membrane rafts.

The interactions between many types of differing molecules and biological membranes underlie much of the cell biology, physiology, and pathology. During the course of such interactions a number of physical factors play important roles and may be used to monitor such interactions. Three of the most influential parameters involve the different membrane potentials that have quite separate identities and origins but appear to be a feature of biological membranes (1). The membrane potentials include the transmembrane potential, resulting from a charge gradient across the membrane, the surface potential, arising from the net excess charge present at the membrane surface, and the membrane dipole potential, which has its origin in the molecular dipoles located on the membrane lipid molecules (1, 7, 41).

The involvement of the transmembrane and surface potentials in many biological processes is fairly well established (2); the role of the membrane dipole potential, however, has only very recently become apparent. Recent studies implicate the dipole potential in the interactions of a number of different molecular species with membranes, such as gramicidin (3, 4), phospholipase A (5), signal sequences (6, 7), and fusion peptides (8).

A number of methods have evolved to monitor the interaction of molecules with membranes, one such method introduced by our laboratory exploits the variations of the magnitude of the membrane surface potential resulting from the attachment of charged molecules to the membrane (1). This technique makes use of indicators (usually fluorescent) that are located precisely at the membrane-solution interface and respond to the magnitude of the membrane surface potential. One particularly useful indicator, fluorescein phosphatidylethanolamine (FPE),[1] has been utilized in our and several other laboratories (*e.g.* Ref. 9) to report the interactions of many types of molecules with membranes (1).

In a similar manner, variations of the membrane dipole potential can also be used to report the membrane binding and insertion of molecules by recording the fluorescence emission of di-8-ANEPPS-labeled membranes as a result of the ratio of two excitation wavelengths. The dual-wavelength ratiometric method complements the FPE-based technique as it facilitates the measurement of the membrane interactions of uncharged molecules (7, 8). The monitoring of the membrane dipole potential as a means to determine intermolecular interactions, however, has been used mainly with model membrane systems, and apart from a pilot study published from our laboratory (10), no comprehensive studies have been reported with living cells. In the present paper, therefore, we demonstrate the possibility of using both FPE and di-8-ANEPPS in a complementary way with model membranes and with living cells with a view to revealing the role of the membrane dipole potential in affecting important cellular processes. In model membrane systems the capacity of sterols such as cholesterol and 6-ketocholestanol to affect the magnitude of the membrane dipole potential is well established (7, 8, 11, 12). On the other hand, cholesterol is an important component of the membrane lipid domains known as "rafts" (13, 14), and removal of cholesterol from cellular membranes following treatment with cyclodextrins or alteration of its behavior by utilizing filipin, amphotericin, and other compounds has been widely reported as a useful method to disrupt "detergent-resistant" membrane microdomains (14–16). Although the importance of cholesterol for the phase separation

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[1] The abbreviations used are: FPE, fluorescein phosphatidylethanolamine; HIV, human immunodeficiency virus; PC, phosphatidylcholine; PS, phosphatidylserine; KC, ketocholestanol; PLV, phospholipid vesicles; Pgp, P-glycoprotein; di-8-ANEPPS, 1-(3-sulfonatopropyl)-4-[β[2-(di-*n*-octylamino)-6-naphthyl]vinyl]pyridinium betaine.

processes involved in the formation of the lipid domains has been well recognized (13, 14, 17, 18), any role of the molecular dipoles associated with sterols and its influence on the electrostatic properties of membranes for the organization and function of the raft's components has not yet been explored in detail.

We report studies on the interactions of a model molecule, saquinavir, an HIV protease inhibitor with Caco-2 cells, a hybridoma established as a model system to study the properties of intestinal epithelia. Oral bioavailability seems to be very limited mainly due to poor solubility, first pass hepatic metabolism, and poor intestinal permeation (19–21). Saquinavir and other HIV protease inhibitors have been described as substrates, inhibitors, or modulators of a number of systems such as the multidrug resistance MDR1 gene product (P-glycoprotein) and the multidrug resistance-associated protein (22–28). The lipidic composition of the membrane has been reported to be important for the activity of P-glycoprotein, particularly the sterol content (29). The transporter has also been suggested to be associated with rafts and "caveolae" (30–32), structures especially rich in cholesterol and sphingolipids.

In the present paper, we present evidence supporting the possibility that saquinavir interacts with a membrane receptor and the fact that such interaction is greatly influenced by the magnitude of the membrane dipole potential. Removal of cholesterol with β-cyclodextrin leads to a decrease in the magnitude of the dipole potential and a reduced binding of saquinavir to the membrane. These results suggest a role for the dipole potential in the regulation of the interaction of molecules with membranes.

MATERIALS AND METHODS

Egg phosphatidylethanolamine (PE) and egg phosphatidylcholine (PC) were purchased from Lipid Products. A pressure extruder bomb for model membrane preparation was obtained from Lipex BM Inc., Vancouver, Canada. Polycarbonate filters (100-nm pore size) were purchased from Nucleopore Filtration Products (Pleasonton, CA). 6-Ketocholestanol (KC) and phloretin were purchased from Sigma. FPE was synthesized as previously described according to Wall *et al.* (33). Di-8-ANEPPS was purchased from Molecular Probes (Leiden, The Netherlands). Saquinavir was purchased from Roche Molecular Biochemicals. Dulbecco's modified Eagle's medium, fetal bovine serum, glutamine, non-essential amino acid, penicillin-streptomycin, and HEPES were purchased from Life Technologies, Inc. Trypsin and Me_2SO were purchased from Sigma. EDTA was purchased from Fisons Scientific Equipment. *Staphylococcus aureus* membrane vesicles were kind gifts of Barry Middleton and Prof. Paul Williams.

Preparation of Large Unilamellar Phospholipid Vesicles (PLVs)—PC and phosphatidylserine (PS) dissolved in chloroform were mixed in a round bottom flask and dried under a stream of oxygen-free argon gas by rotary evaporation until a thin film was formed. The lipid film was rehydrated with 1 ml of 280 mM sucrose, 10 mM Tris, pH 7.4 (sucrose buffer). The resulting multilamellar solution was frozen and thawed 5 times and finally extruded 10 times through 25-mm diameter polycarbonate filters with pores 100 nm in diameter. This resulted in a monodisperse, unilamellar suspension of phospholipid vesicles (34).

Labeling of PLVs and S. aureus Vesicles with FPE and di-8-ANEPPS—Phospholipids were labeled exclusively in the outer bilayer leaflet with FPE as described in Cladera and O'Shea (1). Briefly, the unilamellar vesicles were incubated with FPE dissolved in ethanol (never more than 0.1% of the total aqueous volume) at 37 °C for 1 h in the dark. Any remaining unincorporated FPE was removed by gel filtration on a PD10 Sephadex column equilibrated with the appropriate buffer. Such a procedure leads to the incorporation of 30–50% of the externally added FPE to the preformed membrane vesicle. Furthermore, there was no observed transmembrane flipping of the FPE, at least over time scales of 1 week. The FPE-liposomes were stored at 4 °C until use.

PLVs and *S. aureus* vesicles were labeled with di-8-ANEPPS by adding 1 μM dye (from a stock solution in ethanol) in 280 mM sucrose, 10 mM Tris, pH 7.4.

Cell Culture—Caco-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (heat-activated), 1% glutamine, 1% non-essential amino acids, 2% penicillin-streptomycin, and 2% HEPES. Cells were grown in 25-cm^3 tissue culture flasks, incubated at 37 °C in a humidified atmosphere of 5% CO_2,95% air. The culture medium was changed every 2 days, and the semiconfluence monolayers were subcultured with trypsin-EDTA (0.02% trypsin, 10 mM EDTA).

Labeling of Caco-2 Cells with FPE and Di-8-ANEPPS—Caco-2 cells were trypsinized with trypsin-EDTA and counted with the hemocytometer following the trypan blue exclusion technique to assess cell viability. Caco-2 cells were suspended in the sucrose-based medium (sucrose buffer) (10 mM Tris, 280 mM sucrose, pH 7.4). A volume of FPE in chloroform/methanol at a ratio of 10 μg of FPE:2×10^6 cells was placed in a tube, and the organic solvent evaporated under a steam of argon gas followed by re-solvation with 15 μl of ethanol. The cell suspension was then added to the FPE suspension, and the mixture was gently agitated before incubation in the dark for 45–60 min at 37 °C. The unincorporated FPE was removed by centrifugation at $2500 \times g$ for 5 min in sucrose buffer.

Cells were labeled with di-8-ANEPPs as follows: 1 μM dye was added into a suspension containing 40,000 cells/ml. The mixture was incubated for 2½ h at 36 °C. After this period very small increments in the intensity of the excitation spectra could still be detected as a consequence of the dye incorporating into the membrane. This variation however did not lead to any spectral shift that could compromise the difference spectra of the kind originating from the peptide after normalization (see Fig. 3).

Caco-2 cells (approximately 40,000 cells/ml) were treated with 15 μM KC or 15 μM phloretin for 1 h after they were labeled with di-8-ANEPPS. After this time no additional spectral shifts due to the variation of the dipole potential were detected.

Removal of Cholesterol from Caco-2 Cells—Caco-2 cells in culture were treated overnight with 30 mM methyl-β-cyclodextrin, the cholesterol chelator (35). After this period methyl-β-cyclodextrin was washed by centrifugation, and the cells were labeled with di-8-ANEPPS as described above.

Fluorescence Measurements—Fluorescence time courses were obtained by adding the desired amount of compound to 2-ml lipid suspensions (200 μM lipid or 40,000 cells/ml) on a SLM-AMINCO series 2 spectrofluorometer. For FPE experiments excitation and emission wavelengths were set at 490 and 518 nm, respectively. Di-8-ANEPPS excitation spectra were obtained by excitation at the indicated wavelengths while the emission intensity was measured at 580 nm (7, 11). Dual-wavelength recordings with the di-8-ANEPPS dye were obtained by exciting the samples at two different wavelengths (450 and 520 nm) and measuring their emission intensity ratio, $R_{(450/520)}$, at 580 nm (7, 11). Any contribution of light-scattering to the fluorescence signals was corrected from identical recordings with unlabeled membranes. The cumulative amplitudes of the FPE or di-8-ANEPPS fluorescence signals were plotted against the saquinavir concentration and fitted to standard binding models (36) according to the following equations.

$$\text{Observed signal} = 100\%\ \text{signal} \times [\text{saquinavir}] \times K_d + [\text{saquinavir}] \quad \text{(Eq. 1)}$$

$$\text{Observed signal} = 100\%\ \text{signal} \times [\text{saquinavir}]^n/(K_d)^n + [\text{saquinavir}]^n \quad \text{(Eq. 2)}$$

where K_d is the affinity of the peptide for the membrane in concentration units and n is the Hill coefficient.

RESULTS

The Interaction of Saquinavir and Calcium Ions with Phospholipid Membranes—The interaction of positively charged molecules such as calcium ions and polylysine with model PLVs promotes an increase in the fluorescence of FPE as illustrated in the *inset* of Fig. 1. This property is only manifest when FPE is incorporated into the membrane (1). In a similar manner, serial additions of saquinavir also result in an increase of the fluorescence intensity caused by the interaction of the positively charged form of saquinavir with the membrane. At pH 7.4, ~25% of the overall population of saquinavir molecules are positively charged (p*K* 6.89). A complete titration of the phospholipid membranes with saquinavir is illustrated in the *lower inset* of Fig. 1. The cumulative signal changes as a result of this titration (corrected for any contribution from the solvent addi-

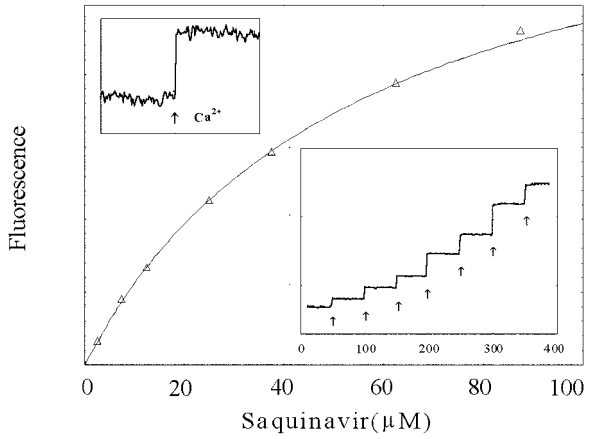


FIG. 1. **Fluorescence variation of FPE-labeled PLVs as a function of saquinavir concentration.** The binding profile was derived from the time course fluorescence variations caused by addition of saquinavir to FPE-PLVs (*lower inset*); the *arrows* indicate successive additions of saquinavir (2.5, 5, 5, 12.5, 12.5, 25, and 25 μM). The experimental data were fitted to Equation 1 (*solid line*). The *upper inset* illustrates the fluorescence variation caused by the addition of 10 mM $CaCl_2$. The lipid concentration was 200 μM. Temperature was 37 °C.

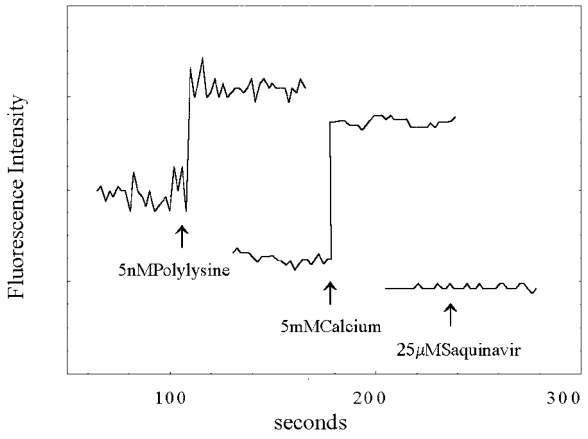


FIG. 2. **Time course of the fluorescence variation of FPE-labeled Caco-2 cells upon addition of 10 mM $CaCl_2$, 200 nM polylysine, and 25 μM saquinavir.** Cell concentration was 2×10^5/ml. Other conditions were as in Fig. 1.

tion) were analyzed according to a number of binding models (main figure). The simplest such model found to be an adequate description of the hyperbolic binding profile is given by Equation 1 and yields a dissociation constant of 50 μM.

The Interaction of Saquinavir, Ca^{2+}, and Polylysine with FPE-labeled Caco-2 Cells—Fig. 2 shows the interactions of polylysine, Ca^{2+}, and saquinavir with FPE-labeled Caco-2 cells. Both Ca^{2+} and polylysine additions resulted in an increase of the fluorescence intensity consistent with the mode of operation of FPE (1). In contrast to the results obtained with PLVs, shown in Fig. 1, however, saquinavir addition was not found to affect the fluorescence intensity of the FPE-labeled Caco-2 cells. The simplest interpretation for this observation is that little of the positively charged saquinavir becomes bound to the Caco-2 cells. In the event that the uncharged fraction of saquinavir becomes bound to the membrane, this would lead to no signal changes and remain, therefore, unobserved with the FPE measurement system. This possibility is addressed experimentally with another indicator system below.

The Interaction of Saquinavir with Caco-2 Cells Monitored with Di-8-ANEPPS; the Role of the Membrane Dipole Potential in Intermolecular Membrane Interactions—The magnitude of the membrane dipole potential may be monitored using the fluorescent indicator di-8-ANEPPS (7, 11). The response of di-8-ANEPPS to variations of the dipole potential, however, involves a spectral shift rather than, as in the case of FPE, a simple intensity change. An example of the measurement of such a spectral shift for both Caco-2 cells and phospholipid membranes labeled with di-8-ANEPPS is illustrated in Fig. 3*A*. The fluorescence difference spectra were obtained respectively by subtracting the excitation spectra of phospholipid membranes and Caco-2 cells before and after their exposure to saquinavir. For the difference spectra to reflect only the spectral shift, the areas of the excitation spectra were normalized to the same integrated intensity before subtraction (7, 37). In both cases, the difference spectra show a minimum below 450 nm and a maximum around 520 nm. These features coincide with those of difference spectra obtained after treatment of membranes with compounds known to decrease the dipole potential (7, 11 and see Fig. 5*B*). The addition of saquinavir to the model and cellular membranes, therefore, appears to promote a decrease in the magnitude of the dipole potential.

The variation of the dipole potential as a function of time can

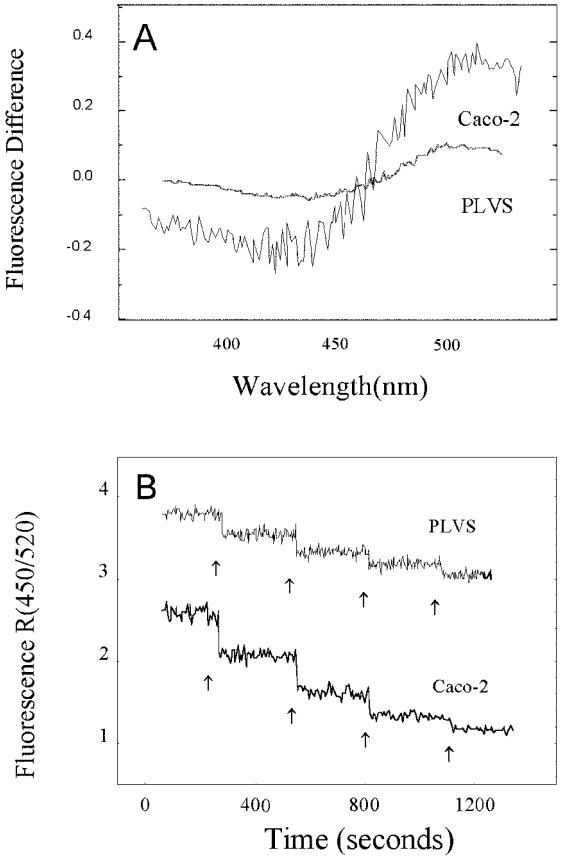


FIG. 3. *A*, di-8-ANEPPS-labeled PLVs and Caco-2 cell excitation difference spectra. The spectra were obtained by subtracting the excitation spectra before the addition of saquinavir from the excitation spectra after the addition of saquinavir. Before subtraction the spectra were normalized to the integrated areas so that the difference spectra would reflect only spectral shifts. *B*, time course variation of the fluorescence ratio $R_{(450/520)}$ measured with the dual-wavelength method. Each *arrow* indicates the addition of 12.5 μM saquinavir. Dye concentration was 1 μM. Other conditions were as in Figs. 1 and 2.

also be monitored using a double wavelength ratiometric method that involves the measurement of the emission ratio at two different excitation wavelengths, $R_{(450/520)}$. This parameter reflects the spectral shift caused by the variation of the dipole potential. Fig. 3*B* shows that the addition of saquinavir to both PLVs and Caco-2 cells causes, as expected from the observation of the difference spectra, a decrease of the ratio $R_{(450/520)}$.

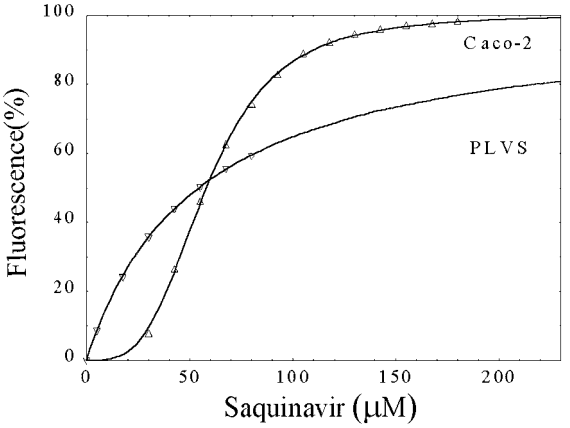
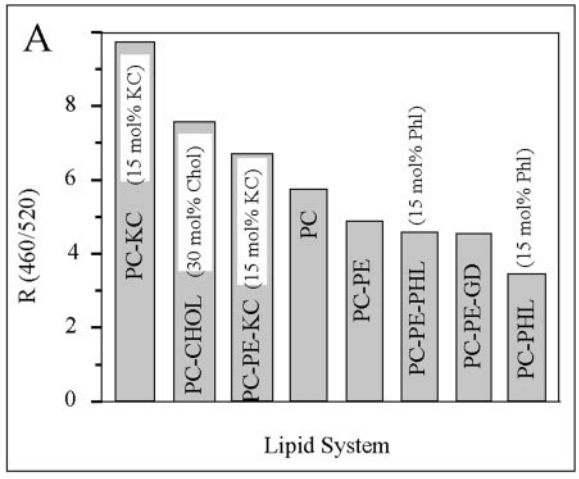


FIG. 4. **Fluorescence change of PLVs and Caco-2 cells labeled with di-8-ANEPPS as a function of saquinavir concentration.** Experimental conditions were as in Fig. 3. Fitting of the experimental points to Equation 1 (PLVs) and Equation 2 (Caco-2 cells) is shown as *solid lines*.

The Membrane Affinity of Saquinavir as Revealed by Di-8-ANEPPS Fluorescence—The changes of the value of $R_{(450/520)}$ following the serial addition of saquinavir to each membrane system may be plotted cumulatively as illustrated in Fig. 4. The experimental data were analyzed according to a number of binding models (*e.g.* as in Fig. 1). The interaction of saquinavir with the phospholipid membranes fits a hyperbolic single binding site model and yields a dissociation constant close to 50 μM. The interaction of saquinavir with the Caco-2 cells, however, was best described by a sigmoidal binding profile (see Equation 2) indicating that there are elements of cooperativity in the binding process (Hill coefficient $n = 3$ from Equation 2). The resultant binding profiles of each membrane system are plotted together in Fig. 4, in which the total extents of the signal change have been normalized to 100% on the ordinate scale for clarity.

Modulation of the Caco-2 Cell Membrane Dipole Potential by 6-Ketocholestanol and Phloretin; Comparison with Phospholipid Membrane Systems—The magnitude of the dipole potential, a property of membranes originating from the molecular dipoles present on the lipid molecules, depends on the composition of the lipid bilayer, as shown in Fig. 5*A* for a range of model membrane compositions. It is clear from the measurement of $R_{(450/520)}$ that compounds such as cholesterol, KC, and phloretin may be used to increase or decrease, respectively, the magnitude of the dipole potential in membranes. Fig. 5*B* shows the fluorescence difference spectrum obtained by subtracting the normalized fluorescence profiles of untreated Caco-2 cell membranes, *i.e.* cells with a "normal" dipole potential, from cells treated with 15 μM KC. This difference spectrum, with a minimum at 520 nm and maximum at 450 nm, is blue-shifted (equivalent to a increase in the ratio $R_{(450/520)}$) following treatment with KC. The result of treating Caco-2 cells with phloretin produces a red-shifted difference spectrum (equivalent to a decrease of $R_{(450/520)}$). These compounds, therefore, may be used with cells to alter the poise of the dipole potential in the same way as with model membrane systems (7, 11).

Modulation of the Caco-2 Cell Membrane Dipole Potential by 6-Ketocholestanol and Phloretin; Effects on the Interactions of Saquinavir—The interaction of saquinavir with the Caco-2 cell membrane affects the magnitude of the membrane dipole potential as indicated by the difference spectrum and the decrease of $R_{(450/520)}$ shown in Fig. 3. Fig. 6*A* illustrates the time evolution of $R_{(450/520)}$ upon saquinavir addition to Caco-2 cell membranes treated with KC and phloretin as compared with untreated membranes. It is noteworthy that the initial level of



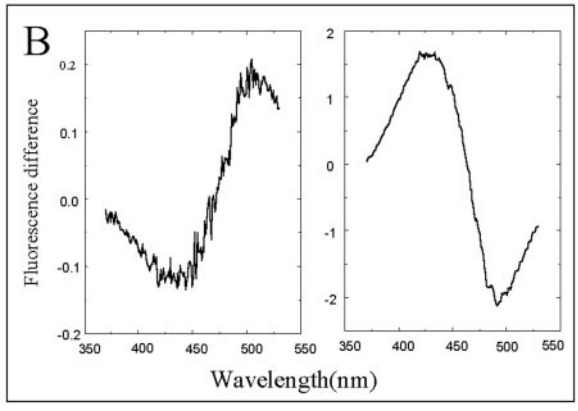


FIG. 5. *A*, dependence of the magnitude of the dipole potential, expressed as the fluorescence ratio $R_{(460/520)}$, on membrane composition. *PC-KC*, 85 mol% PC:15 mol% KC; *PC-CHOL*, 70 mol% PC:30 mol% cholesterol; *PC-PE-KC*, 35 mol% PC:50 mol% PE:15 mol% KC; *PC*, 100 mol% PC; *PC-PE*, 50 mol% PC:50 mol% PE; *PC-PE-PHL*, 35 mol% PC:50 mol% PE:15 mol% phloretin; *PC-PE-GD*, 25 mol% PC:50 mol% PE:25 mol% ganglioside; *PC-PHL*, 85 mol% PC:15 mol%. *B*, di-8-ANEPPS-labeled Caco-2 cell fluorescence difference spectra. *Left panel*, excitation spectrum of cells treated with 15 μM phloretin minus excitation spectrum of untreated cells. *Right panel*, excitation spectrum of cells treated with 15 μM KC minus excitation spectrum of untreated cells. Subtraction procedure and other conditions were as in Fig. 3.

the fluorescence ratio $R_{(450/520)}$ is different for each of the membrane systems in accordance with the previous section (Fig. 5*A*).

The extent of the change following challenge with saquinavir is different in each of the membrane systems utilized, as illustrated in Fig. 6*A*, with respect to the untreated Caco-2 cells. The amplitude of the signal decrease is larger for Caco-2 cells treated with KC and smaller for cells treated with phloretin.

Fig. 6*B* illustrates the comparison between the binding profiles of saquinavir to untreated cells and cells treated with KC and phloretin. Each binding process, following correction for any contribution from Me_2SO, was analyzed and found to be best described as a sigmoidal profile indicating a level of cooperativity. The index of cooperativity in each case (*i.e.* the Hill coefficient) was found to be close to 3. Compared with untreated membranes, the magnitude of the binding capacity, however, was dramatically increased in membranes treated with KC and marginally decreased in membranes treated with phloretin.

Effect of 6-Ketocholestanol, Cholesterol, and Phloretin on the Binding of Saquinavir to PLVs—The effect of phloretin and KC

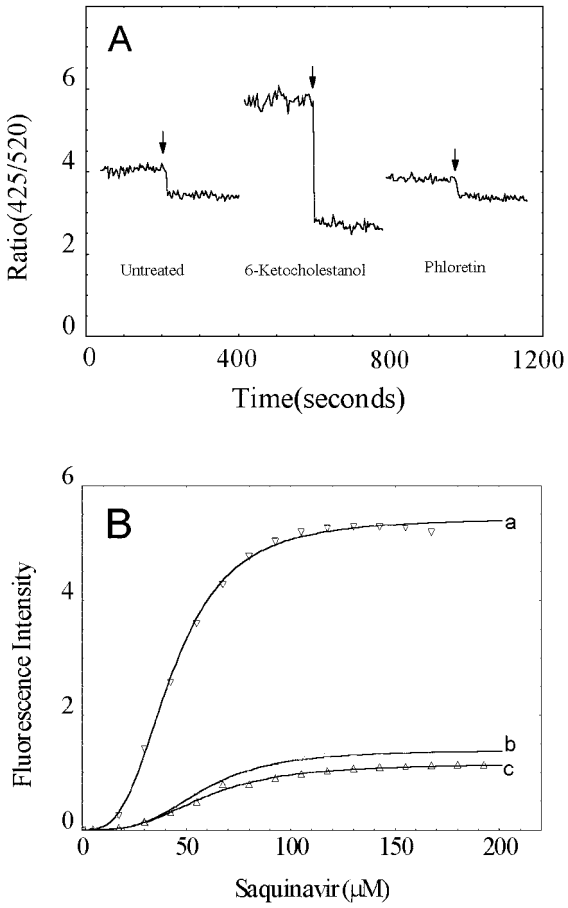


FIG. 6. *A*, effect of phloretin and KC on the fluorescence variation of di-8-ANEPPS-labeled Caco-2 cells upon saquinavir addition (12.5 μM). B, variation of the ratio $R_{(450/520)}$ as a function of saquinavir concentration. *a*, Caco-2 cells supplemented with 15 μM KC; *b*, Caco-2 cells; *c*, Caco-2 cells supplemented with 15 μM phloretin. The initial value of $R_{(450/520)}$ has been normalized to 0. Fitting of the data to Equation 2 is shown as a *solid line*.

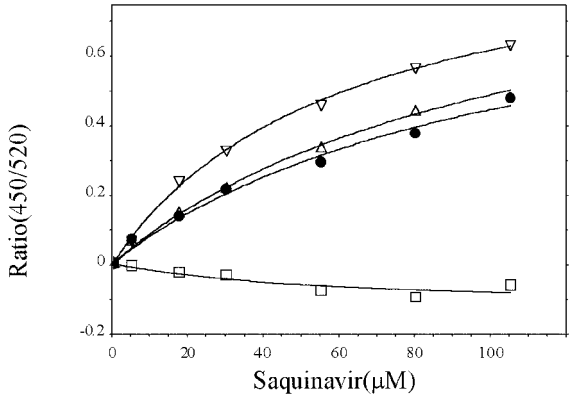


FIG. 7. **Effect of phloretin, KC, and cholesterol on the binding of saquinavir to di-8-ANEPPS-labeled PLVs.** The variation of the ratio $R_{(450/520)}$ as a function of saquinavir concentration is presented: PLVs supplemented with 15 mol% KC (*downward triangles*), PLVs (*full circles*), PLVs supplemented with 20 mol% cholesterol (*upward triangles*), PLVs supplemented with 15 mol% phloretin (*squares*). The initial value of $R_{(450/520)}$ has been normalized to 0. Fitting of the data to Equation 1 is shown as a *solid line*.

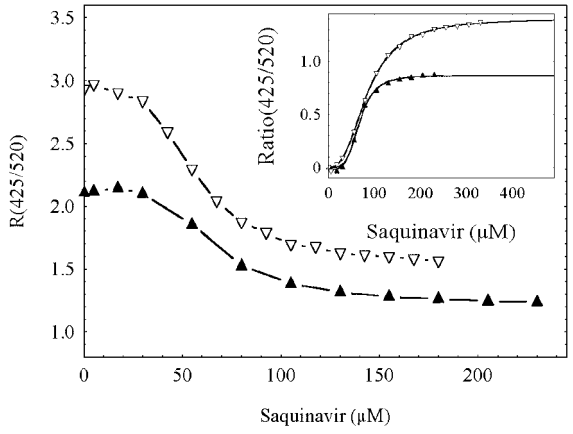


FIG. 8. **Variation of the fluorescence ratio $R_{(450/520)}$ as a function of saquinavir concentration for di-8-ANEPPS-labeled Caco-2 cells (*downward triangles*) and di-8-ANEPPS-labeled Caco-2 cells pretreated with methyl-β-cyclodextrin to remove cholesterol from the cell membrane (*upward triangles*).** The *inset* represents both profiles after normalizing the initial value of $R_{(450/520)}$ to 0 to compare the difference in the amplitude of the fluorescence variation at saturating saquinavir concentrations. Data shown in the *inset* have been fitted to Equation 2 (*solid lines*).

together with the observed effects of cholesterol on the binding of saquinavir to PLVs is illustrated in Fig. 7. As was the case with Caco-2 cells, the modification of the dipole potential influences the saquinavir binding capacity. Cholesterol is shown to act in a manner similar to KC but to a much lesser extent. The data in this case, however, were best fitted to a hyperbolic binding profile (Equation 1) with no indications of cooperative interactions.

Effect of Partially Removing Cholesterol from the Caco-2 Cells on the Membrane Binding of Saquinavir—It has been demonstrated above that cholesterol may be utilized in a similar manner as KC to increase the membrane dipole potential and that its presence in the model lipidic bilayers affects the binding capacity of saquinavir. On this basis it was considered worthwhile to determine the effect that the removal of cholesterol from the native Caco-2 cells using methyl-β-cyclodextrin (14–16, 35) had on the membrane interactions of saquinavir. Treatment of the cells with methyl-β-cyclodextrin is known to remove 40–50% of the cholesterol present in the cell membrane (16). Fig. 8 illustrates how the magnitude of the membrane dipole potential is smaller for cells that have been treated with the methyl-β-cyclodextrin (lower value of the initial *R* parameter). This is consistent with a significant reduction in the amount of cholesterol in the plasma membrane since the presence of cholesterol leads to an increase in the magnitude of the dipole potential (11). The *inset* in the figure, in which the initial values of $R_{(450/520)}$ have been normalized to 0 to facilitate the comparison of the fluorescence changes, shows that the binding capacity (maximal fluorescence change) is reduced when saquinavir binds to cells treated with methyl-β-cyclodextrin. The affinity of the compound for the membrane and the sigmoidal binding profile, however, remain the same.

Saquinavir Binding to S. aureus Membrane Vesicles and Effect of Colchicine on the Binding to Caco-2 Cells—Finally, we considered the possibility of measuring the interaction of saquinavir with membranes with no sterols while still representing a membrane system abundant in nature that possesses the potential to bind molecules such as saquinavir. With this in mind *S. aureus* membranes were utilized because they are known to possess drug resistance systems within their cell membrane, a system homologous to the eucaryotic mutidrug resistance and to the LmrA of *Lactobacillus lacti* (38, 39). *S. aureus* membranes also do not contain sterols. The binding of saquinavir to *S. aureus* membrane vesicles was found to exhibit a sigmoidal profile, very similar to that measured for Caco-2 cells, as shown in Fig. 9. This observation and the results presented above clearly show that cooperativity in the binding of saquinavir does not depend on the presence of cholesterol or

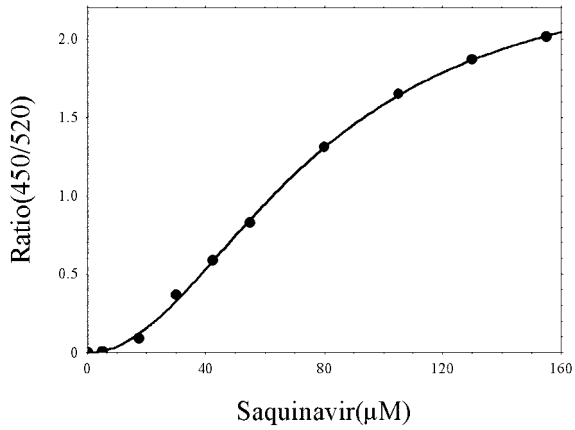


FIG. 9. **Fluorescence change of *S. aureus* membrane vesicles labeled with di-8-ANEPPS as a function of saquinavir concentration.** Experimental conditions were as in Fig. 3. Fitting of the experimental points to Equation 2 is shown as a *solid line*.

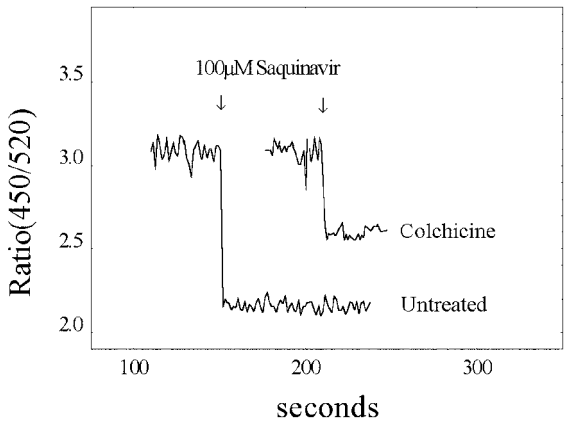


FIG. 10. **Time course of the fluorescence variations of di-8-ANEPPS-labeled Caco-2 cells in the absence and presence of 10 mM colchicine in the medium.** Experimental conditions were as in Fig. 3.

the magnitude of the dipole potential but rather on the protein content of the membranes. This is compelling evidence that strongly suggests the involvement of a membrane receptor. The identity of such a membrane receptor is likely to be the P-glycoprotein as there is good evidence that saquinavir interacts with this membrane protein (22–28). This possibility is further strengthened by observations that competitive inhibition of saquinavir binding is observed with colchicine, an established Pgp substrate known to affect the conformation of Pgp in the concentration range between 1 and 10 mM. Fig. 10 indicates that the presence of colchicine at levels known to inhibit partially the action of Pgp significantly reduces the interaction of saquinavir with Caco-2 cells. This implies that saquinavir interacts with the plasma membrane Pgp and underlies the sigmoidal nature of the cell binding profile indicated in Fig. 4.

DISCUSSION

The fluorescent indicators FPE and di-8-ANEPPS have been used previously in the study of the relationship between membrane potentials and the interaction of molecules with biological membranes. In the case of FPE, the labeling of cell membranes has been characterized for several cellular systems (1); studies involving di-8-ANEPPS, however, have been mostly undertaken using model membrane systems (7, 8, 10, 11, 37, 40, 41). The present study outlines the use of monitoring the dipole potential with the ratio-fluorescence of di-8-ANEPPS in combination with FPE to report the membrane electrostatic surface potential changes, for the complete interrogation of the interactions between macromolecules and cellular membranes. The results clearly emphasize the significance of the membrane dipole potential in the interaction of the HIV1-protease inhibitor saquinavir with the plasma membrane of Caco-2 cells.

Fluorescence measurements with FPE-labeled and di-8-ANEPPS-labeled phospholipid model membranes (PLVs) and Caco-2 cells show that saquinavir interacts with both membrane systems (Figs. 1 and 4). The interaction of saquinavir with FPE-labeled cells, however, does not cause any fluorescence variation, whereas it clearly increases the fluorescence of FPE-labeled PLVs (Figs. 1 and 2). On the other hand, the FPE-labeled cells are sensitive to the interactions of other positively charged compounds known to have a high affinity for the membrane, such as calcium ions and polylysine (Fig. 2). This indicates that the dye is correctly incorporated into the cellular membrane with the fluorophore located precisely at the membrane surface. In fact in our laboratory such a calcium response is utilized routinely as a diagnostic for the successful incorporation of the dye into the membrane (1, 33, 36). To explain the lack of response of FPE-labeled cells when challenged with saquinavir, as compared with PLVs, however, it is necessary to take other factors into consideration. In particular, the amount of phospholipid membrane presented by the Caco-2 cell suspension compared with that presented by the PLV suspension is very different. This difference between the model membrane and the cellular systems has recently been described to be important also for understanding the HIV gp41 fusion peptide interaction with biological membranes (10). Following the approximation described in this work it may be calculated that the respective phospholipid surface area is at least 10^4 smaller in the Caco-2 cell experimental system. Thus, positively charged saquinavir (representing approximately 25% of the total saquinavir population), which clearly interacts with model membranes, may also interact with the lipidic part of the Caco-2 cell membrane. However because this represents such a small contribution to the fluorescence it remains undetectable under such conditions.

Identical studies undertaken with di-8-ANEPPS shown in Fig. 3, however, indicate that saquinavir interacts with the plasma membrane of Caco-2 cells, as such interaction leads to a decrease of the membrane dipole potential, which can be used to obtain the binding profile. As di-8-ANEPPS does not discriminate between the uncharged and charged portions of the saquinavir population, additional information needs to be brought to bear if statements are to be made on the identity of the molecular species that is interacting with the membrane. From our previous conclusions that very little of the charged population of saquinavir binds to the Caco-2 cells and given that the sensitivity of the FPE measurement system is much greater than that of the di-8-ANEPPS, it seems most likely that the uncharged components of the saquinavir population become bound to the Caco-2 cells. In other words the di-8-ANEPPS signal changes reside in the binding of saquinavir molecules that are uncharged as otherwise binding of charged saquinavir would be observed by the (more sensitive) FPE-labeled Caco-2 cells, electrostatic surface potential measurement system.

A binding model incorporating a single population of binding sites describes satisfactorily the binding to PLVs (Fig. 4), whereas in the case of Caco-2 cells, a sigmoid profile with a Hill coefficient close to 3 offers the best fit of the experimental data. Binding of saquinavir to the phospholipid components of the cell membrane would be anticipated to produce a hyperbolic binding profile as in the case of phospholipid model membranes. Such a difference in the shape of the binding profiles between the different membranes is a strong indication of the

possibility that the binding of saquinavir to Caco-2 cells has its origins in a receptor-mediated process rather than solely phospholipid membrane binding. In line with this, saquinavir like other HIV-1 protease inhibitors is known to be a substrate of the MDR1 multidrug transporter and other drug resistance systems such as the multidrug resistance-associated protein (19, 22–28).

The sigmoidal nature of the binding profiles does not appear to depend on the cholesterol content of the lipidic bilayer or on the presence of compounds such as phloretin or KC, which affect the magnitude of the dipole potential. The interaction of saquinavir with PLVs always yields hyperbolic binding profiles independently of the membrane composition (Fig. 7), whereas binding to cell or bacterial membranes exhibits sigmoidal profiles (Figs. 6*B* and 9) despite the fact that bacterial membranes are known to contain no sterols. The membranes of *S. aureus*, however, are known to possess membrane proteins involved in drug resistance and homologs of the eucaryotic human MDR1 and the LmrA of *L. lacti* (38, 39). Cooperativity in the binding of saquinavir, therefore, appears to rely on the presence of membrane proteins and indicates that a membrane receptor is involved in the binding process.

On the other hand, it follows from the present results that the initial magnitude of the membrane dipole potential clearly influences the binding of saquinavir (*i.e.* from the total amplitude of the binding plots). As we demonstrate in Fig. 5*A*, the magnitude of the dipole potential is highly dependent on the lipid composition of the membrane. The use of compounds such as cholesterol, KC, or phloretin to poise the dipole potential of either model or cell membranes shows that the higher its initial magnitude (*i.e.* more positive toward the interior of the bilayer (7, 41)) the higher the saquinavir binding capacity, without major changes in the affinity of the compound for the membrane being observed (Figs. 6, 7, and 8).

It is worth emphasizing the fact that cholesterol has a similar but lesser effect on the membrane dipole potential as KC as well as to the binding capacity of saquinavir for both model and cell membranes. Cholesterol is also known to promote phase separation in lipid bilayers, which leads to the formation of microdomain structures "afloat" within the fluid phospholipid bilayer (13) known as rafts. Removal of cholesterol by treatment of cells with cyclodextrins has been extensively reported to disrupt membrane rafts (14–16). Our results, therefore, are consistent with a model in which the binding capacity of saquinavir is enhanced when the receptor is located in rafts as a consequence of the increased magnitude of the dipole potential in these cholesterol-rich patches compared with that in the fluid phase of the bilayer. The results reported here strongly suggest a possible role of the membrane dipole potential in the interaction of molecules with rafts and the important biological processes associated with them (13, 14).

Finally, the binding competition experiments (Fig. 10), although preliminary, point toward P-glycoprotein as the identity of the saquinavir membrane receptor. Colchicine is a rather hydrophilic Pgp substrate. Druley *et al*. (42) reported that colchicine can modify the conformation of Pgp in the concentration range between 1 and 10 mM. This is consistent with the level of colchicine used to interfere with the binding of saquinavir to Caco-2 cells in the present study.

The indications in the present paper that the membrane dipole potential may influence the interaction of saquinavir with model and cell membranes shed new light on how some physical properties of membranes may be utilized to control cellular phenomena. Molecular dipoles within membranes may underlie the behavior of protein systems within membrane microdomains. On this basis, the strategy employed in the present paper seems appropriate for future experiments to study the interaction of a number of signal molecules with "raft-associated" receptor systems. In our laboratory this is being pursued by applying imaging techniques to the more localized interactions of such molecules with similarly localized receptors on the cell surface. It also seems possible using the technologies described above that comparisons between otherwise similar bacterial and eucaryotic membrane systems can also be addressed.

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REFERENCES

1. Cladera, J., and O'Shea, P. (2001) in *Protein-Ligand Interactions* (Harding and Chowdery, eds) pp. 169–200, Oxford University Press, Oxford, UK
2. Clementz, T., Christiansson, A., and Wieslander, A. (1988) in *Physiological Regulation of Membrane Fluidity*, pp. 41–74, Alan R. Liss, Inc., New York
3. Rokitskaya, T. L., Atonenko, Y. N., and Kotova, E. A. (1997) *Biophys. J.* **73,** 850–854
4. Shapovalov, V., Kotova, E. A., Rokitskaya, T. I., and Antonenko, Y. N. (1999) *Biophys. J.* **77,** 299–305
5. Maggio, B. (1999) *J. Lipid Res.* **40,** 930–939
6. Voglino, L., McIntosh, T. J., and Simon, S. A. (1998) *Biochemistry* **37,** 12241–12252
7. Cladera, J., and O'Shea, P. (1998) *Biophys. J.* **74,** 2434–2442
8. Cladera, J., Martin, I., Ruysschaert, J. M., and O'Shea, P. (1999) *J. Biol. Chem.* **274,** 29951–29959
9. Bryson, E. A., Rankin, S. E., Carey, M., Watts, A., and Pinheiro, T. J. (1999) *Biochemistry* **38,** 9758–9767
10. Cladera, J., Martin, I., and O'Shea, P. (2001) *EMBO J.* **20,** 19–26
11. Gross, E., Bedlack, R. S., and Loew, L. M. (1994) *Biophys. J.* **67,** 208–216
12. Clarke, R. J., and Kane, D. J. (1997) *Biochim. Biophys. Acta* **1323,** 223–239
13. Simons, K., and Ikonen, E. (1997) *Nature* **387,** 569–572
14. Simons, K., and Toomre, D. (2000) *Nature Rev. Mol. Cell. Biol.* **1,** 31–39
15. Hansen, G. H., Niels-Christiansen, L. L., Thorsen, E., Immerdal, L., and Danielsen, E. M. (2000) *J. Biol. Chem.* **275,** 5136–5142
16. Luker, G. D., Pica, C. M., Sampath Kumer, A., Covey, D. F., and Piwnica-Worms, D. (2000) *Biochemistry* **39,** 7651–7661
17. Anderson, R. G. W. (1998) *Annu. Rev. Biochem.* **67,** 199–225
18. Brown, D. A., and London, E. (2000) *J. Biol. Chem.* **275,** 17221–17224
19. Aungst, B. J. (1999) *Adv. Drug Del. Rev.* **39,** 105–116
20. Watkins, P. B. (1997) *Adv. Drug Del. Rev.* **27,** 161–170
21. Gottesman, M. M., Hrycyna, C. A., Schoenlein, P. V., German, U. A., and Pastan, I. (1995) *Annu. Rev. Genet.* **29,** 607–649
22. Lee, C. G. L., Gottesman, M. M., Cardarelli, C. O., Ramachandra, M., Jeang, K. T., Ambudkar, S. V., Pastan, I., and Dey, S. (1998) *Biochemistry* **37,** 3594–3601
23. Gutmann, H., Fircker, G., Drewe, J., Toeroek, M., and Miller, D. S. (1999) *Mol. Pharmacol.* **56,** 383–389
24. Kim, R. B., Fromm, M. F., Wandel, C., Leake, B., Wood, A. J., Roden, D. M., and Wilkinson, G. R. (1998) *J. Clin. Invest.* **101,** 289–294
25. Miller, D. S., Nobmann, S. N., Gutmann, H., Teoroek, M., Drewe, J., and Fricker, G. (2000) *Mol. Pharmacol.* **58,** 1357–1367
26. Profit, Eagling, V. A., and Back, D. J. (1999) *AIDS* **13,** 1623–1627
27. Jones, K., Hoggard, P. G., Sales, S. D., Khoo, S., Davey, R., and Back, D. J. (2001) *AIDS* **15,** 675–681
28. Srinivas, R. V., Middlemas, D., Flynn, P., and Fridland, A. (1998) *Antimicrob. Agents Chemother.* **42,** 3157–3162
29. Ferte, J. (2000) *Eur. J. Biochem.* **267,** 277–294
30. Zegers, M. M. P., and Hoekstra, D. (1998) *Biochem. J.* **336,** 257–269
31. Demeule, M., Jodoin, J., Gingras, D., and Beliveau, R. (2000) *FEBS Lett.* **466,** 219–224
32. Lavie, Y., Fiucci, G., Czarny, M., and Liscovitch, M. (1999) *Lipids* **34,** (suppl.) S57–S63
33. Wall, J., Golding, C., van Veen, M., and O'Shea, P. (1995) *Mol. Membr. Biol.* **12,** 183–192
34. Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986) *Biochim. Biophys. Acta* **858,** 161–168
35. Kilsdonk, E. P. C., Yancey, P., Stoudt, G., Bangerter, F. W., Johnson, W. J., Philips, M. C., and Rothblat, G. H. (1995) *J. Biol. Chem.* **270,** 17250–17256
36. Wall, J., Ayoub, F., and O'Shea, P. (1995) *J. Cell Sci.* **108,** 2673–2682
37. Montana, V., Farkas, D. L., and Loew, L. M. (1989) *Biochemistry* **28,** 4536–4539
38. Putman, M., van Veen, H. W., and Konings, W. N. (2000) *Microbiol. Mol. Biol. Rev.* **64,** 672–693
39. van Veen, H. W., and Konings, W. N. (1998) *Biochim. Biophys. Acta* **1365,** 31–36
40. Loew, L. M., Scully, S., Simpson, L., and Waggoner, A. S. (1979) *Nature* **281,** 497–499
41. Brockman, H. (1994) *Chem. Phys. Lipids* **73,** 57–59
42. Druley, T. E., Stein, W. D., Ruth, A., and Roninson, I. B. (2001) *Biochemistry* **40,** 4323–4331

Effects of the Membrane Dipole Potential on the Interaction of Saquinavir with Phospholipid Membranes and Plasma Membrane Receptors of Caco-2 Cells

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