ABSTRACT

Verapamil is one of the frequently prescribed calcium channel blockers used in the treatment of hypertension and angina pectoris. Results of evaluations of the therapy have led to reports of toxic effects. This study presents several evidences that verapamil affects human cells. Scanning electron microscopy observations of intact human erythrocytes indicated that they underwent morphological alterations as increasing verapamil concentrations starting from 5 μM changed their discoid normal shape, and finally to hemolysis. Fluorescence spectroscopy on isolated unsealed human erythrocyte membranes confirmed these outcomes. In fact, the assays showed that verapamil induced a significant increase of the anisotropy parameters and a moderate one of the generalized polarization, indicative of enhanced order at the acyl chain and polar head regions of the erythrocyte membrane lipid bilayer. X-ray diffraction experiments on dimyristoylphosphatidylcholine and dimyristoylphosphatidylethanolamine bilayers, classes of the major phospholipids present in both outer and inner sides of the erythrocyte membrane, respectively showed that verapamil perturbed the polar head and acyl chain regions of both lipid bilayers. These interactions were found to be stronger with DMPC bilayers. On the other hand, human SH-SY5Y neuroblastoma cells incubated with verapamil suffered a sharp decrease of cell viability.

INTRODUCTION

Verapamil hydrochloride (Fig. 1) is a phenylalkylamine-derivate calcium-channel blocking agent. Chemically, it is a basic (log K = 9.1) and highly hydrophobic compound (log P = 9.1). It is used in the treatment of hypertension and angina pectoris, and has also been shown to be effective in the treatment of tachyarrhythmia, variant angina, and cardiomyopathy. While verapamil is one of the frequently prescribed calcium channel blockers, certain results of the therapy evaluations have led to reports of toxic effects and overdoses at concentrations above 1000 ng/ml. The exact molecular mechanism of verapamil capacity to perturb the structures of DMPC and DMPE was assessed by X-ray diffraction, intact human erythrocytes were observed by scanning electron microscopy and isolated unsealed human erythrocyte membranes (IUM) were studied by fluorescence spectroscopy. These systems and techniques have been used in our laboratories to determine the interaction with and the membrane-perturbing effects of other drugs.

MATERIALS AND METHODS

X-ray diffraction studies of DMPC and DMPE multilayers

Verapamil capacity to perturb the structures of DMPC and DMPE multilayers was evaluated by X-ray diffraction. Synthetic DMPC (lot 80H-8371, MW 677.9) and DMPE (lot 084K-1676, MW 635.9) from Sigma (St. Louis, MO), and verapamil.HCl (MW 491.1) from Aldrich (Milwaukee, WI) were used without further purification. About 2 mg of each phospholipid was introduced into 1.6 mm diameter special glass capillaries (Glas-Technik 8371, MW 677.9) and DMPE (lot 084K-1676, MW 635.9) from Sigma (St. Louis, MO), and verapamil.HCl (MW 491.1) from Aldrich (Milwaukee, WI) were used without further purification. About 2 mg of each phospholipid was introduced into 1.6 mm diameter special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany, which were then filled with 150 μl of (a) distilled water and (b) aqueous solutions in a range of concentrations. The specimens were X-ray diffracted after 30 min incubation at 30°C and 60°C with DMPC and DMPE, respectively. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuKa radiation from a Bruker KBr270 (Karlsruhe, Germany) was used. The relative reflection intensities were obtained in an MBrAua PSD-50M linear position-sensitive detector system (Garching, Germany); no correction factors were applied. Experiments were performed at 18°C ± 1°C, which is below the main phase transition temperature of both DMPC and DMPE. Higher temperatures would have induced transitions to more fluid phases making the detection of structural changes harder. Each experiment was performed in triplicate and in case of doubts additional experiments were carried out.

Fig. 1. Structural formula of verapamil hydrochloride.
Scanning electron microscope (SEM) studies of intact human erythrocytes

A range of verapamil concentrations was prepared in order to interact in vitro with red blood cells by incubating erythrocyte suspensions obtained from a healthy human donor not receiving any pharmaceutical treatment. Blood was obtained by puncture of the ear lobe; 100 μL were received in an eppendorff tube containing 10 mL of heparin (5000 UI/mL). 900 mL of saline solution (NaCl 0.9%, pH 7.4) was added. The tube was centrifuged (1000 rpm x 10 min), the supernatant was discarded and replaced by the same volume of saline solution; the whole process was repeated three times. Fractions of this stock of red blood cells suspension (RBCS) in saline solution were placed in each of one eppendorff tubes to prepare (A) the control, by mixing 150 μL saline solution plus 100 mL RBCS, and (B) a range of concentrations by mixing 100 μL of RBCS in saline with 150 μL of each verapamil concentration. All the samples were then incubated in an oven at 37 °C for 1 h, period in line with the larger effects of studied compounds on red cell shape.14 Afterwards, they were fixed for 12 h at 4 °C with 900 μL 2.5% glutaraldehyde in distilled water, reaching a final fixation concentration of about 2.4%. Finally, previously centrifuged samples at 1000 rpm were washed in distilled, re-suspended in 200 μL of distilled water; drops of each one were placed on Al glass cover stubs, air-dried at room temperature, gold coated and examined in a scanning electron microscope (JEOL JSM-6380LV, Japan).

Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM).

Another approach used to analyze the influence of verapamil on the physical properties of human erythrocyte membranes was the measurement of two fluorescence parameters: anisotropy (r) and generalized polarization (GP) by fluorescence spectroscopy. This issue was achieved by using two different fluorescent probes: 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan). Isolated human erythrocyte membranes (IUM) were prepared with human blood obtained from healthy male donors according to the method of Dodge et al.15 Briefly, by means of both isotonic and hypotonic phosphate buffered saline (PBS), erythrocytes were separated from plasma, lysed and then washed in order to obtain the unsealed membranes, which were stored in isotonic solution at -20 ºC. IUM were incubated with DPH and Laurdan by addition of small aliquots of concentrated solution of the probe in tetrahydrofuran and ethanol, respectively, at 37 ºC for about one hour. Fluorescence spectra and anisotropy measurements were performed on a K2 steady-state and time-resolved spectrofluorometer (ISS Inc., Champaign, IL, USA) interfaced to computer, using the corresponding ISS software. Temperature was monitored before and after each measurement by a digital thermometer (Omega Engineering Inc., Stamford, CT, USA). Samples were measured using 5 mm path-length square quartz cuvettes. Anisotropy measurements were made in the L configuration using Glan Thompson prism polarizers in both exciting and emitting beams. Using an excitation wavelength of 360 nm, the emission was measured with a WG-420 Schott high-pass filter (Schott, Mainz, Germany) with negligible fluorescence. DPH fluorescence anisotropy (r) was calculated according to the definition \( r = (I_H / I_V) / I_H \), where \( I_H \) and \( I_V \) are the corresponding parallel and perpendicular emission fluorescence intensities with respect to the vertically polarized excitation light.14 Laurdan fluorescence spectral shifts were quantitatively evaluated using the generalized polarization (GP) concept \( P = (I_H - 1) / (I_H + 1) \), where \( I_H \) and \( I_V \) are the emission intensities at the blue and red edges of the emission spectrum, respectively. With excitation at 360 nm these intensities have been measured at the emission wavelengths of 440 and 490 nm, which correspond to the emission maxima of laurdan in the gel and liquid crystalline phases, respectively.16 Verapamil was incorporated in IUM suspensions by addition of adequate aliquots in order to obtain different concentrations in the 0-1 mM range. These samples were then incubated for 10-15 minutes at 37 ºC. Blanks were prepared using samples without probes. Data presented in Table 1 represent mean values and standard error of 15 measurements in two independent samples.

Viability studies on human neuroblastoma cells SH-SY5Y

SH-SY5Y human neuroblastoma cells were purchased from ECACC (European Collection of Cell Culture, Salisbury, UK). SH-SY5Y with a cell cycle of 23 h were cultured in DMEM/ F12 (Gibco, Carlsbad, CA USA) medium containing 15% (v/v) fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO), 100 units/ml penicillin (Gibco, Carlsbad, CA USA) and streptomycin (100 mg/ml; Gibco, Carlsbad, CA USA), at 37 ºC with 5% CO\(_2\) in a humidified atmosphere (90% humidity). The medium was replaced every 2 days. 0.25% Trypsin-EDTA solution and phosphate buffered saline (PBS) were obtained from Sigma Aldrich (DMPC). Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Sigma Aldrich St. Louis, MO). Briefly, SH-SY5Y cells were seeded into 24-well plates (at a density of 10 x 10\(^3\) cells per well, in 1.5 mL medium). 2% FBS medium containing verapamil at a concentration range from 1 nM to 0.5 mM was added to the cells for 1, 6, 24 and 48 h. The drug was prepared as a 43 mM stock solution in bi-distilled water. MTT (5 mg/ml) was added to each well and incubated in the dark at 37 ºC for 3 h followed by cells lysis and spectrophotometric measurement at 550 nm (Microplate SPECTRAmax\(^\text{A}^\text{TM}\) at 550). The MTT solution was carefully decanted off, and formazan was extracted from the cells with 1 mL of acidic isopropanol (0.04 M HCl in absolute isopropanol) in each well. Colour was measured with a 96-well ELISA plate reader. All MTT assays were performed three times in duplicate. All readings were compared with the control, which represented 100% viability. Data regarding MTT assay were performed using T-test and Primer software, and values were reported as highly statistically significant if \( P < 0.01 \) and statistically significant if \( P < 0.05 \). Results are presented as mean ± standard deviation.

RESULTS

![Fig. 2. Microdensitograms from X-ray diffraction patterns of (A) dimyristoylphosphatidylcholine (DMPC), and (B) dimyristoylphosphatidylethanolamine (DMPE) multilayers in water and aqueous solutions of verapamil hydrochloride; (LA) and (WA) correspond to low- and wide-angle reflections, respectively.](image-url)

Fig. 2A exhibits the results obtained by incubating DMPC with water and verapamil. As expected, water altered the structure of DMPC, as its bilayer repeat (phospholipid bilayer width plus the layer of water) increased from about 55 Å in its dry crystalline form to 64 Å when immersed in water, and its low-angle reflections (indicated as LA), which correspond to DMPC polar terminal groups, were reduced to only the first two orders of the bilayer repeat. On the other hand, only one strong reflection of 4.2 Å showed up in the wide-angle region (indicated as WA), which corresponds to the average distance between fully extended acyl chains organized with rotational disorder in pllaonal packing. These results were consistent with those obtained for DMPC bilayers. Fig. 2A discloses that after exposure to 50 μM verapamil, there was a considerable decrease of DMPC reflection intensities, which became almost negligible with 100 μM, an effect observed in both the low- and wide-angle DMPC reflections. From these results, it can be concluded that verapamil produced a significant structural perturbation, affecting both the polar head and acyl chain regions of DMPC bilayers and, as a consequence, a disruption of the in-pllaonal packing. These results are in line with those obtained for DMPE multilayers, as all the DMPE peaks were significantly decreased with increasing verapamil concentrations.
Scanning electron microscope (SEM) studies of intact human erythrocytes

SEM examinations of human erythrocytes incubated with verapamil indicated that the drug induced different changes to the normal biconcave morphology of the red blood cells (Fig. 3A). Fig. 3B shows that when incubated with 5 μM most of the cells presented stomatocytosis, an altered condition in which the erythrocytes show a cup-shaped form with evagination of one surface and a deep invagination of the opposite; Fig. 3C shows that when treated with 500 μM the majority of the cells are stomatocytes, there are several knizocytes (red blood cells with two or three concavities due to indentations in the cell membrane), spherostomatocytes (cells with a visible change towards spheroid morphology with lightly or minor cupped profiles), and echinocytes (the erythrocytes show a spiny configuration, exhibiting blebs or protuberances in their surfaces); with 1.0 mM the majority of the cells are knizocytes (Fig. 3E), while 5 mM verapamil, the highest assayed concentration, produced the lysis of most of the cells (Fig. 3F).

Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM).

Verapamil concentration-dependent effects on IUM were explored at two different depths of the lipid bilayer: at the hydrophilic/hydrophobic interface level, estimated from the laurdan fluorescence spectral shift through the generalized polarization (GP) parameter, and in the deep hydrophobic core, determined by the DPH steady-state fluorescence anisotropy (r). Table 1 shows that the incorporation of 1 μM and 10 μM verapamil to IUM induced a 22 % and 26 % increase, respectively, of the anisotropy (r) at 37°C, implying an ordering effect in the hydrocarbon chain region of the lipid bilayer in the liquid crystalline state. Table 1 also shows no effects in the general polarization (GP) parameter, implying that verapamil, in these concentrations did not affect IUM polar head groups. However, 1 mM (data not shown) induced a 12 % increase in the general polarization (GP) parameter, implying a polarity and or a molecular dynamics decrease, presumably due to a moderate ordering of IUM polar head groups.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>r (DPH)</th>
<th>GP (laurdan)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.167 ± 0.014</td>
<td>0.246 ± 0.012</td>
</tr>
<tr>
<td>1</td>
<td>0.203 ± 0.013</td>
<td>0.246 ± 0.011</td>
</tr>
<tr>
<td>10</td>
<td>0.210 ± 0.018</td>
<td>0.246 ± 0.012</td>
</tr>
</tbody>
</table>

Viability studies on human neuroblastoma cells SH-SY5Y

The effects of verapamil on human SH-SY5Y neuroblastoma cells were assayed at a concentration range from 1 nM to 0.5 mM. Cells were incubated for 1, 6, 24 and 48 h. As it can be appreciated in Fig. 4, a highly statistically significant decrease of cell viability was observed with 0.5 mM verapamil treatment. This toxic effect was immediate, since it was evident only after 1 h, and it was not recovered because it remained after 48 h. The other tested concentrations were not able to produce any significant effect after 1, 6, and 24 h; only after 48 h a significant decrease of cell viability was observed with 0.1 mM verapamil.

These findings clearly showed that from 100 μM and 10 μM lies the threshold between toxic and non toxic concentration of verapamil.

DISCUSSION

The data herein reported demonstrates that verapamil affects human erythrocytes. Scanning electron microscopy observations of intact human erythrocytes indicated that they underwent morphological alterations as increasing verapamil concentrations from 5 μM changed their discoid normal shape to stomatocytes, knizocytes, spherostomatocytes, and to hemolysis with 5 mM verapamil. According to the bilayer couple hypothesis, the morphological changes induced in erythrocytes by foreign molecules are due to the differential expansion of their two monolayers. Thus, speculated shapes (echinocytes) are induced when the added compound is inserted in the outer monolayer, whereas cup shapes (stomatocytes) arise when the compound accumulates in the inner monolayer. The X-ray diffraction experiments performed on bilayers made up of DMPC and DMPE, classes of the major phospholipids present in the outer and inner sides of the erythrocyte membrane, respectively showed that verapamil disordered the polar head and acyl chain regions of both DMPC and DMPE, these interactions being stronger with DMPC bilayers. DMPC and DMPE differ only in their terminal amino groups, these being ‘N(CH₃)₃’ in DMPC and ‘NH₂’ in DMPE. Moreover, both molecular conformations are very similar in their dry crystalline phases with the hydrocarbon chains mostly parallel and extended, and the polar head groups lying perpendicularly to them. However, the gradual hydration of DMPC results in water filling the highly polar interbilayer spaces with the

Fig. 4. Cytotoxicity assay in SH-SY5Y cells after treatment with verapamil hydrochloride in a range of concentrations. Neuroblastoma redox activity was measured by MTT assay. The data represented are mean ± SD of two individual experiments, each done in duplicate. *P<0.05, **P<0.01 vs control.
resulting increase of their width. This phenomenon allows the incorporation of verapamil into DMPC bilayers with the resulting disruption of its structure. Chemically verapamil hydrochloride is a highly hydrophobic compound (log P<sub>ow</sub> = 9.1; therefore, it is possible that it intercalates into the membrane lipid bilayer located into the vicinity of the polar/apolar membrane interface. On the other hand, DMPE molecules pack tighter than those of DMPC due to their smaller polar groups and higher effective charge, resulting in a very stable bilayer system that is not significantly affected by water. This organization of the preferential interaction of verapamil with DMPC, a class of lipid mainly containing r values, indicative of enhanced order at acyl chain regions of the erythrocyte membrane. Fluorescence spectroscopy on IUM at 37 ºC confirmed these results.

However, these effects were much milder than those observed in the bilayer system that is not significantly affected by water. This organization was further supported by the observation that protein function will be affected by the structure of the lipid bilayer. It must be considered that alteration of the normal biconcave cell morphology. It must be considered that alteration of the normal biconcave shape of red blood cells increases their resistance to entry into capillaries, which could contribute to a decreased blood flow, loss of oxygen, and tissue damage through microvascular occlusion. Functions of ion channels, receptors and enzymes immersed in cell membrane lipid moieties also might be affected. According to it, some cationic amphipaths produce a rapid scrambling of the erythrocyte bilayer with phosphatidylcholines (PC) and sphingomyelins (SM) moving inward while phosphatidylethanolamines (PE) moves outward along with phosphatidylserines (PS). Thus, the interaction of verapamil with PC in the inner monolayer would lead to stomatocytosis, an effect that can be produced by as little as 0.6% enrichment of the cytoplasmic monolayer. However, interactions of verapamil with proteins located in the outer monolayer of the erythrocyte membrane cannot be disregarded. In fact, the ordering effect in the hydrocarbon chain region of the lipid bilayer detected in the fluorescence experiments occurred at very low drug concentrations with no subsequent significant effect. Therefore, it might be possible that the drug also interacted with the erythrocyte membrane proteins, with an ordering effect at the lipid protein interface.

Human SH-SYSY neuroblastoma cells incubated with 0.5 mM verapamil suffered a sharp decrease of cell viability. The primary pharmacological effect of verapamil is to block L-type Ca<sup>2+</sup> channel. It has already been demonstrated that this drug, at massive concentrations, could alter Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger transport activity. This could partly explain the dramatic decrease of cellular viability at the highest assayed verapamil concentration (0.5 mM).

Amazingly, the therapeutic range for plasma verapamil concentrations and the relationship of plasma concentration to clinical response and toxicity have not been clearly established. Toxic effects and overdoses at concentrations above 1000 ng/ml (equivalent to approx. 2 μM verapamil) have been reported in serum. A typical I.V. dose during cardiac care would be approximately 5-10 mg of verapamil hydrochloride, which would be equivalent to a circulating dose of about 4 μM; however, its oral use in angina involves doses of 120-160 mg 2-3 times daily; overdosing on just 10 tablets would result in circulating concentration of around 325 μM. Our experimental results showed that 5 μM verapamil altered the shape of erythrocytes inducing stomatocytosis, 1 μM affected the anisotropy of DPH in IUM, and 50 μM almost completely perturbed the bilayer structure of DMPC. As reported by Watts and Handy, 50 μM and higher verapamil concentrations caused hemolysis in vitro in a time dependent manner; although they could not identify the precise mechanism of this effect, they suggested a drug action on several ion-transport pathways and non-specific oxidative membrane damage.

Our experimental findings are certainly of interest as they demonstrated that verapamil interacts with the human erythrocyte membrane affecting the cell morphology. It must be considered that alteration of the normal biconcave shape of red blood cells increases their resistance to entry into capillaries, which could contribute to a decreased blood flow, loss of oxygen, and tissue damage through microvascular occlusion. Functions of ion channels, receptors and enzymes immersed in cell membrane lipid moieties also might be affected. Accordingly to Lee, integral membrane proteins are not rigid entities around which the lipid bilayer distorts enough as to provide the strongest interactions. Rather, both the lipid and proteins will distort to provide the best interactions, with the result that protein function will be affected by the structure of the surrounding lipid bilayer. These findings may also provide a new insight into the possible mechanism of action of verapamil.