Phospholipids Bilayers as Molecular Models for Drug-Membrane Interactions. The Case of the Antiepileptics Phenytoin and Carbamazepine

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Summary: With the aim to better understand the molecular mechanisms of the interaction of phenytoin and carbamazepine with cell membranes we utilized a well-established model consisting in intact human erythrocytes, isolated unsealed human erythrocyte membranes (IUM) and molecular models of its membrane. The latter consisted of bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidyl-ethanolamine (DMPE), representative of phospholipid classes respectively located in the outer and inner monolayers of erythrocytes and other cell membranes. This report presents the following evidence that phenytoin and carbamazepine interact with membrane phospholipids: a) X-ray diffraction and fluorescence spectroscopy showed that both drugs preferentially interacted with DMPC; b) in IUM, the drugs induced a disordering effect on the polar head groups and acyl chains of the eryhrocyte membrane lipid bilayers; c) electron microscopy observations of human erythrocytes showed the echinocyte formation, an effect due to phenytoin and carbamazepine insertion in the outer monolayer of the red cell membrane.

Keywords: carbamazepine; cell membrane; lamella; phenytoin; phospholipids bilayer

Introduction

In the course of *in vitro* systems search for the toxicity screening of therapeutic drugs various cellular models have been applied to examine their adverse effects in isolated organs. This article describes the effects of two antiepileptic drugs, phenytoin and carbamazepine at the cell membrane level. The cell membrane is an assembly of proteins and lipids that separate inside from outside, protecting the cell interior. The membrane is also involved in a variety of indispensable cellular functions. It is responsible for the selective transport of molecules and ions into and out of the cell in the extensive network, and for the traffic between organelles. Without exception, these activities depend on, and are influenced by the physical *milieu* provided by the molecules making up the membrane bilayers. Changes in the physical and chemical environment of the cell membranes have a direct effect on the membrane structure with serious effects on the cell functions.[1–2] Most biological membranes possess an asymmetric trans-bilayer distribution of phospholipids.[3] Thus, for instance, most eukaryotic plasma membranes present a high percentage of the phospholipids sphingomyelins and phosphatidylcholines in the outer monolayer whereas the inner one is generally richer in phosphatidyl-ethanolamine, phosphatidylserines and phosphatidylinositols.

Carbamazepine is widely used in the treatment of epilepsy, neuralgic pain and bipolar affective disorders.[4] The pharmacological mechanism by which it exerts its

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clinical effects is not well established[5], although its molecular basis of action has been ascribed to voltage-dependent inhibition of Na^+ channel currents, being more pronounced at more depolarized membrane potentials[6] The use of this drug is limited by its high toxicity.[7] It has also been reported that people treated with carbamazepine presented hematological alterations,[8] anemia,[9] minimum changes of fatty acid composition of erythrocyte membranes,[10] pure red cell aplasia,[11] and changes of the human erythrocyte glutathione and glutathione peroxidase.[12] However, despite the ample use of carbamazepine and numerous reports of its toxicity, its effects on the human erythrocyte membrane have been rarely studied.[10] Phenytoin is also an antiepileptic agent used with much clinical success for over 60 years. Its mechanism of action, however, is still open to interpretation. The best evidence also hinges on the inhibition of voltage-sensitive Na^+ channels in the plasma membrane of neurons undergoing seizure activity.[13] This was an additional reason for studying the binding affinities of phenytoin and carbamazepine with cell membranes.

With the aim to better understand the molecular mechanisms of the interaction of phenytoin and carbamazepine with cell membranes we utilized a well-established model consisting in intact human erythrocytes and molecular models of its membrane. The latter consist of bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidyl-ethanolamine (DMPE), representative of phospholipid classes respectively located in the outer and inner monolayers of erythrocytes and other cell membranes.[3] Erythrocytes were chosen because although less specialized than many other cell membranes the functions they perform, such as active and passive transport, and the production of ionic and electric gradients, allow them to be considered representative of the plasma membrane in general. The capacity of these drugs to interact with the intact human erythrocyte membrane was determined by scanning electron microscopy (SEM). The antiepileptics ability to perturb the isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV) was examined by fluorescence spectroscopy, while their interaction with the bilayer structures of DMPC and DMPE was studied by X-ray diffraction. These techniques have been used in our laboratories to determine the interaction with and the membrane-perturbing effects of local anesthetics,[14–15] antiarrhythmic,[16] and anticancer drugs.[17–18]

Materials and Methods

Reagents

Synthetic DMPC (lot 80H8371, A grade, MW 677.9), DMPE (lot 13H83681, A grade, MW 635.9), phenytoin (lot 39H0752, 99% purity, MW 252.3) and carbamazepine (lot 80K1549, 99% purity, MW 236.3) from Sigma (MO, USA) were used without further purification.

X-Ray Diffraction of Phospholipids Multilayers

About 2 mg of DMPC and DMPE were each mixed in 2.0 mm diameter glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany) with 200 μL of aqueous phenytoin solutions (concentration range 1 mM to 10 mM). In the case of carbamazepine, the same amount of each lipid was mixed in the glass capillaries with the corresponding weight of the drug in order to attain DMPC:carbamazepine and DMPE: carbamazepine powder mixtures in the molar ratios of 10:1, 5:1, 2:1, and 1:1, and these were then filled with about 200 μL of distilled water. The specimens thus prepared were X-ray diffracted after incubation for 24 h in flat-plate cameras with 0.25 mm diameter glass collimators provided with rotating devices. The blanks consisted of pure samples of each phospholipid with excess water. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuKα radiation from a Bruker Kristalloflex 760

(Karlsruhe, Germany) X-ray generator was used. The relative reflection intensities on films were measured by peak integration using Bio-Rad GS-700 densitometer (Hercules, CA, USA) and Molecular Analyst/PC image software; no correction factors were applied. The experiments were performed at $17 \pm 2\,^{\circ}C$, which is below the main transition temperatures 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 repeated at least twice.

Fluorescence Measurements of Isolated Unsealed Human Erythrocyte Membranes (IUM) and Large Unilamellar Vesicles (LUV)

The influence of phenytoin and carbamazepine on the physical properties of IUM and DMPC LUV was examined by fluorescence spectroscopy using DPH and laurdan (Molecular Probe, Eugene, OR, USA) fluorescent probes. DPH is widely used as a probe for the hydrophobic regions of the phospholipid bilayers because of its favorable spectral properties. Its steady-state fluorescence anisotropy measurements were used to investigate the structural properties of IUM and DMPC LUV as it provides a measure of the rotational diffusion of the fluorophor, restricted within a certain region such as a cone, due to the lipid acyl chain packing order. Laurdan, an amphiphilic probe, has a high sensitivity of excitation and emission spectra to the physical state of membranes. With the fluorescent moiety within a shallow position in the bilayer, laurdan provides information about the polarity and/or molecular dynamics at the level of the phospholipid glycerol backbone. The quantification of the laurdan fluorescence spectral shift was effected using the generalized polarization (GP) concept.[19] Erythrocytes were separated from heparinized venous blood samples obtained from normal casual donors by centrifugation and washing procedures. IUM were prepared by lysis according to Dodge et al.[20] DMPC LUV suspended in water were prepared by the extrusion method[21], i.e., by the extrusion of frozen and thawed multilamellar liposome suspensions (final lipid concentration 0.3 mM) through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp., MA, USA) under nitrogen pressure at $10\,^{\circ}C$ above the lipid phase transition temperature. A LUV size distribution of around 400 nm was obtained by dynamic light scattering. DPH and laurdan were incorporated into IUM and LUV by addition of small aliquots of concentrated solutions of the probe in dimethylformamide and ethanol, respectively, and incubated at $37\,^{\circ}C$ for 45 min. Fluorescence spectra and anisotropy measurements were performed on a steady-state and time-resolved spectrofluorometer (ISS Inc., Champaign, IL, USA). Measurements of LUV suspensions were made at $18\,^{\circ}C$ and $37\,^{\circ}C$, and measurements of IUM were made at $37\,^{\circ}C$ using 10 mm path-length square quartz cuvettes. Sample temperature was controlled by an external bath circulator (Cole-Parmer, Chicago, IL, USA) and monitored before and after each measurement using an Omega digital thermometer (Omega Engineering Inc., Stanford, CT, USA). Anisotropy measurements were made in the L configuration using Glan Thompson prism polarizers (I.S.S.) in both exciting and emitting beams. The emission was measured using a WG-420 Schott high-pass filter (Schott WG-420, Mainz, Germany) with negligible fluorescence. DPH fluorescence anisotropy (r) was calculated according to the definition:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the corresponding parallel and perpendicular emission fluorescence intensities with respect to the vertically polarized excitation light.[22] Laurdan fluorescence spectral shifts were quantitatively evaluated using the GP concept (see above) which is defined by the expression $GP = (I_b - I_r)/(I_b + I_r)$, where I_b and I_r are the emission intensities at the blue and red edges of the emission spectrum, respectively. 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.[23] Phenytoin and carbamazepine were incorporated in IUM and LUV suspensions by addition of small aliquots of concentrated solutions and incubated at 18 °C or 37 °C, depending on the work temperature, for ca. 15 min. The IUM were studied at 37 °C because that is the normal temperature at which erythrocytes circulate in humans. In the case of DMPC LUV, the studies were performed at 37 °C for the same reasons described above, and at 18 °C because it is below the transition temperature of DMPC. Blank subtraction was performed in all measurements using labeled samples without probes. The obtained data represented mean values and standard error of ten measurements in two independent samples. Unpaired Student's t-test was used for statistical calculations.

Scanning Electron Microscopy (SEM) of Human Erythrocytes

In vitro interaction of phenytoin and carbamazepine with erythrocytes was attained by incubating red blood cell suspensions derived from healthy human male donors not receiving any pharmacological treatment. Blood samples were obtained by puncture of the ear lobule and by aspiration into a tuberculin syringe without a needle containing 50 units/ml heparin in saline solution (0.9% NaCl). Red blood cells were then centrifuged, washed twice in saline, resuspended in saline containing phenytoin and carbamazepine at final 10 mM and 50 μM concentrations, respectively and incubated for 1 h at 37 °C. Controls were cells resuspended in saline solution without the drugs. Specimens were then fixed overnight at 5 °C by adding one drop of each sample to plastic tubes containing 1 ml of 2.5% glutaraldehyde, washed twice in distilled water, and placed on siliconized Al stubs that were air dried at 37 °C for 30 min and gold coated for 3 min at 13.3 Pascal in a sputter device (Edwards S 150, Sussex, England). Specimens were examined in an Etec Autoscan SEM (Etec Corp., Hayward, CA, USA).

Results and Discussion

X-Ray Diffraction Studies of Phospholipid Multilayers

The molecular interactions of phenytoin and carbamazepine with multilayers of the phospholipids DMPC and DMPE in an aqueous medium were determined by X-ray diffraction. Figure 1A shows a comparison of the diffraction patterns of DMPC alone and of DMPC incubated with phenytoin in the range of 1 mM up to 10 mM. As expected, water altered the structure of DMPC: its bilayer repeat increased from about 55 Å in its dry crystalline form[24] to 64.5 Å when immersed in water, and its reflections were reduced to only the first three orders of the bilayer repeat (bilayer width plus the layer of water). On the other hand, a new and strong reflection of 4.2 Å showed up, whose appearance was indicative of the fluid state reached by DMPC bilayers and corresponded to the average distance between its fully extended acyl chains organized with rotational disorder in hexagonal packing. Addition of 1 mM phenytoin caused only a very slight decrease in the phospholipid reflection intensities. However, phenytoin 3.5 mM and 5 mM caused a marked decrease of these intensities, whereas 10 mM induced the complete disappearance of the low angle reflections [indicated as (a) in the figure], which were replaced by a diffuse halo, and a considerable weakening of the 4.2 Å reflection. This result implies that the drug induced serious molecular disorder in the DMPC bilayer, especially in the region of the polar head groups. Figure 1B shows the results of the interaction of phenytoin with DMPE. The perturbing effect of this compound upon the structure of DMPE bilayers was practically negligible in the low-angle region even at a 10 mM concentration, although phenytoin 5 mM and 10 mM somewhat reduced the phospholipid high angle reflection intensities [indicated as (b)] in the figure. As a matter of fact, these two phospholipids differ only in their terminal amino groups, these being $^{+}N(CH_3)_3$ in DMPC and $^{+}NH_3$ in DMPE.

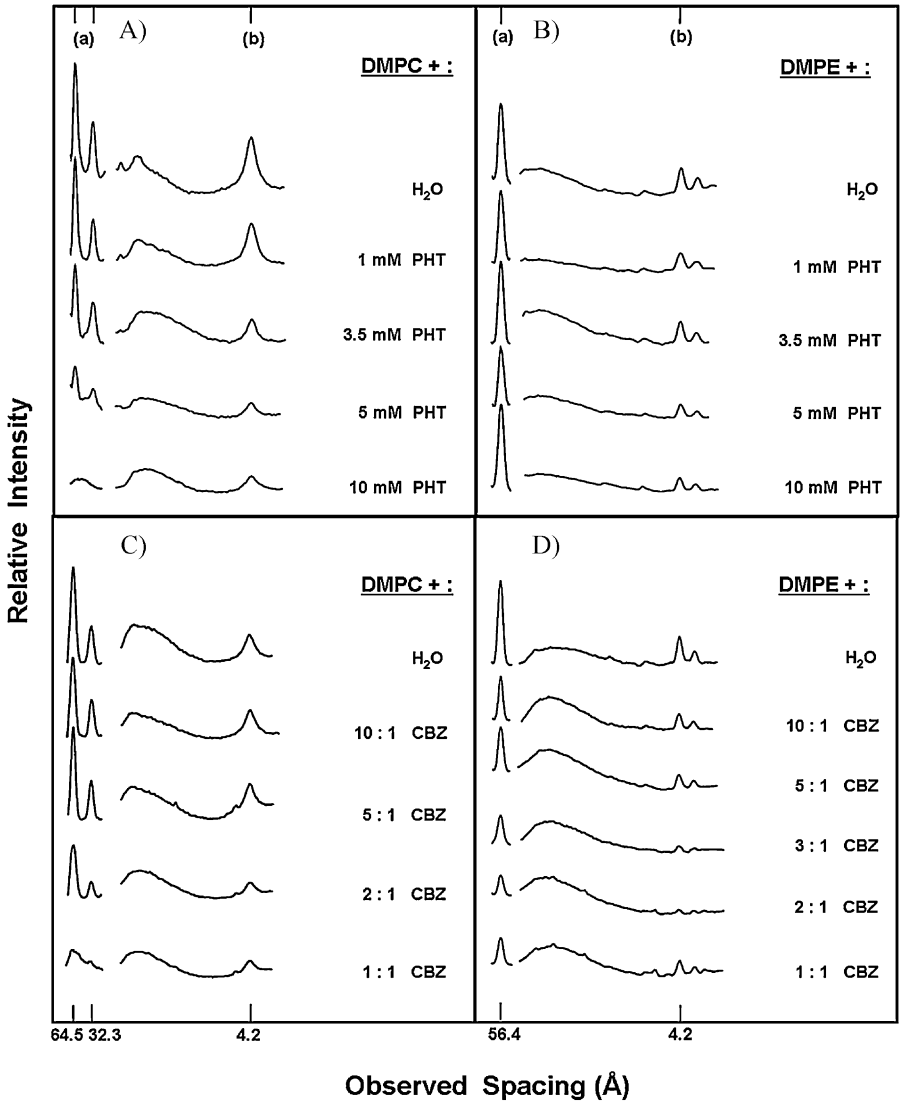

Figure 1.
X-ray diffraction diagrams: (A) Phenytoin (PHT) incubated with dimyristoyl-phosphatidylcholine (DMPC); (B) Phenytoin incubated with dimyristoylphosphatidylethanolamine (DMPE); (C) Carbamazepine (CBZ) incubated with DMPC, and (D) Carbamazepine incubated with DMPE.

Moreover, both molecular conformations are very similar in their dry crystalline phases[24] with the hydrocarbon chains mostly parallel and extended, and the polar groups lying perpendicularly to them.

However, the gradual hydration of DMPC results in water filling the highly polar interbilayer spaces. Thus, its bilayer width increases from 54.5 Å when dry up to about 64 Å when it is fully hydrated. This phenomenon allows the incorporation of phenytoin into DMPC bilayers producing its structural perturbation and almost complete collapse at a 10 mM concentration. On the other hand, DMPE molecules pack tighter than those of DMPC due to their smaller polar group and higher effective charge, resulting in a very stable bilayer

system that is not either affected by water[24] or by a number of drugs.[16] Figure 1C shows a comparison of the diffraction patterns of DMPC alone and of DMPC mixed with carbamazepine in the 10:1, 5:1, 2:1 and 1:1 molar ratios. Addition of carbamazepine in increasing molar ratios caused a monotonically mild decrease in the phospholipid reflection intensities in the low angle region (a), until they practically disappeared at a 1:1 molar ratio, whereas the 4.2 Å still remained although considerably weakened. This result implies that the drug induced molecular disorder in the DMPC bilayer, especially in the region of the polar head groups. Figure 1D shows the results of the interaction of carbamazepine with DMPE. The perturbing effect of this compound upon the structure of DMPE bilayers was much milder than that induced to DMPC since the lipid reflections were still present in their 1:1 molar ratio. From these results it can be concluded that both drugs interacted with DMPC and to a lesser extent with DMPE. The higher effects induced by phenytoin can be explained by its higher solubility in water.

Fluorescence Measurements of Isolated Unsealed Human Erythrocyte Membranes (IUM) and Large Unilamellar Vesicles (LUV)

Phenytoin and carbamazepine concentration-dependent effects on IUM and DMPC LUV 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 GP parameter, and in the deep hydrophobic core, determined by the DPH steady-state fluorescence anisotropy (r). Figure 2A and 2B show that increasing concentrations of phenytoin (0 to 2.0 mM) decreased the r and GP values at 18 °C. Similar measurements performed at 37°C showed that both values remained practically constant in the 0 to 2.0 mM concentration range. It should be taken into account that at 37 °C this lipid is in a much more fluid state than at 37 °C than at 18 °C. Figure 2C and 2D show that the

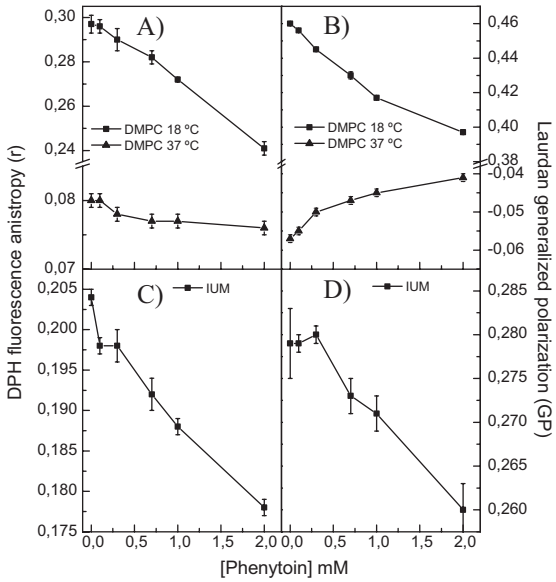

Figure 2.
Effect of Phenytoin on the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the generalized polarization (GP) of laurdan in large unilamellar vesicles (LUV) of dimyristoylphosphatidylcholine (DMPC) and isolated human erythrocyte membranes (IUM). (A) r in DMPC LUV incubated at 18 °C and 37 °C; (B) GP in DMPC LUV incubated at 18 °C and 37 °C; (C) r in IUM incubated at 37 °C; (D) GP in IUM incubated at 37 °C.

incorporation of phenytoin into IUM in the same range of increasing concentrations considerably decreased both the r and GP values of IUM at 37 °C. These results imply that the drug induced structural perturbations in both the acyl chain and polar group packing arrangement of the erythrocyte membrane lipid bilayer.

Figure 3A shows that at 18 °C the incorporation of carbamazepine into DMPC LUV induced a sharp decrease in anisotropy at concentrations higher than 0.75 mM, a result that implies a structural perturbation of the lipid acyl chain region. However, the same experiment performed at 37 °C indicates that the anisotropy value remained practically unchanged, an effect due to the fluid state reached by DMPC at this temperature. Figure 3B shows that carbamazepine also induced a sharp decrease in the general polarization at 18 °C. This result can be interpreted as a disordering effect induced by carbamazepine on DMPC polar groups. In contrast, similar measurements performed at 37 °C showed that there was a mild increase in GP, implying a decrease in polarity and or in a molecular dynamics, presumably due to a moderate ordering of DMPC polar groups. Figure 3C and 3D show that increasing concentrations of carbamazepine (starting at about 0.1 mM), sharply decreased both the r and GP values of IUM at 37 °C, respectively. These results imply that the drug induced structural

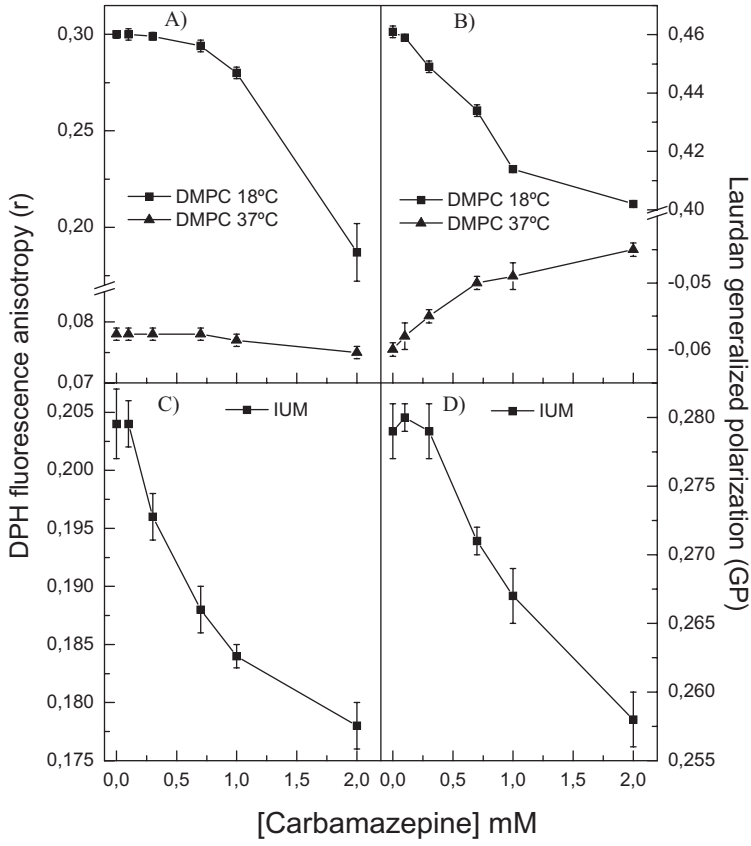

Figure 3.
Effect of Carbamazepine on the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the generalized polarization (GP) of laurdan in large unilamellar vesicles (LUV) of dimyristoylphosphatidylcholine (DMPC) and isolated human erythrocyte membranes (IUM). (A) r in DMPC LUV incubated at 18 °C and 37 °C; (B) GP in DMPC LUV incubated at 18 °C and 37 °C; (C) r in IUM incubated at 37 °C; (D) GP in IUM incubated at 37 °C.

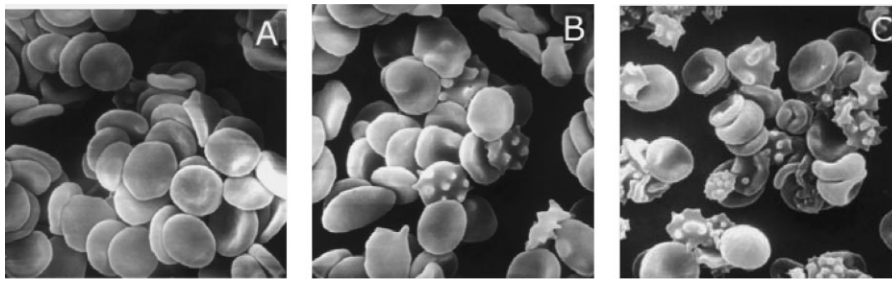

Figure 4.
SEM images of human erythrocytes; (A) control; (B) erythrocytes incubated with 10 mM Phenytoin, and (C) 50 μM Carbamazepine; 2400X.

perturbations in both the acyl chain and polar group packing arrangement of the erythrocyte membrane lipid bilayer.

Scanning Electron Microscopy (SEM) of Human Erythrocytes

SEM observations of human erythrocytes incubated with phenytoin suspensions equivalent to 10 mM revealed that the drug induced changes in the cells' normal shape. As it can be observed in Figure 4B, about half the erythrocytes underwent an echinocytic type of alteration, i.e., developed a form characterized by blebs or protuberances over the cell membrane. On the other hand, human erythrocytes incubated with 50 μM carbamazepine revealed that an average of 85% of the erythrocytes decreased in size and also developed echinocytes (Figure 4C). Some cells (5%) also showed a stomatocytic or cup-shape, i.e., evagination of one surface and a deep invagination of the opposite face, whereas 10% remain unchanged. According to the bilayer couple hypothesis,[25–26] the shape changes induced in erythrocytes by foreign molecules are due to 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 fact that phenytoin and carbamazepine mainly induced echinocyte formation indicates that both drugs were preferentially located in the outer moiety of the red cell membranes. These results agree with those obtained by X-ray diffraction performed on bilayers made up of classes of the major phospholipids present in either the outer or inner sides of the erythrocyte membrane. In fact, they showed that phenytoin and carbamazepine disordered the polar head, and to a lesser extent the acyl chain regions of DMPC and DMPE, where these interactions were considerable stronger with DMPC bilayers. Fluorescence spectroscopy on DMPC LUV at 18 °C confirmed these results. In fact, the assays showed that phenytoin and carbamazepine induced a significant reduction of their PG and r values, indicative of enhanced disorder at the polar head and acyl chain regions of their lipid bilayers.

Conclusion

This report presents the following evidence that phenytoin and carbamazepine interact with membrane phospholipids: a) X-ray diffraction and fluorescence spectroscopy showed that both drugs preferentially interacted with DMPC, class of lipids found in outer monolayers of cell membranes; b) in IUM, the drugs induced a disordering effect on the polar head groups and acyl chains of the eryhrocyte membrane lipid bilayers; c) SEM observations of human erythrocytes showed echinocyte formation, an effect due to the insertion of phenytoin and carbamazepine in the outer monolayer

of the red cell membrane.[24] Plasma therapeutic concentrations of phenytoin are in the 40–80 μM range and toxic effects are encountered at concentrations as high as 0.4 mM.[27] The effects of the drug detected in the present work, including alterations in erythrocyte structure, were observed at much higher concentrations. This indicates that phenytoin cytotoxicity at the cell membrane level is not significant. While serum therapeutic concentrations of carbamazepine are in the 20–40 μM,[28] toxicity has been demonstrated when its serum concentrations exceed 80 μM.[29] Thus, the effects of carbamazepine detected in the present work, particularly alterations in erythrocyte structure, were observed at concentrations of the order of those currently measured in serum when it is therapeutically administered. This finding is certainly of interest as it indicates that carbamazepine cytotoxicity at the cell membrane level is significant. It must also be considered that alteration of the normal biconcave shape of red blood cells into a spiculated form (echinocyte) increases erythrocyte resistance to entry into capillaries;[30] this effect might result in a decrease of blood oxygenation, and explain reported carbamazepine hematological alterations.[8]

Acknowledgements: This work was supported by grants from FONDECYT (1060990).

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