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Phospholipid Bilayers as Molecular Models for Drug-Cell Membrana Interactions. The Case of the Antiinflamatory Drug Diclofenac

Mario Suwalsky,[*1] Marcela Manrique,[1] Fernando Villena,[2] Carlos P. Sotomayor[3]*

Summary: Phospholipids are amphipatic molecules with long hydrophobic acyl chains and zwitterionic polar heads which assemble into different types of molecular aggregates. The most relevant is the bilayer because of its relation with cell membranes, which are very complex entities. For this reason, simpler molecular models based on phospholipids bilayers are widely used. We have determined the bilayer structure of phospholipids located in the outer and inner monolayers of most cell membranes, and use them as molecular models to study the way different chemicals of biological interest interact with cell membranes. We present the results of our studies on the nonsteroidal anti-inflammatory drug diclofenac, from which little is known about its effects on human erythrocytes. This report presents the following evidence that diclofenac interacts with the human red cell membrane: a) X-ray diffraction and fluorescence spectroscopy of phospholipids bilayers show that diclofenac interacts with a class of lipids found in the outer moiety of the erythrocyte membrane; b) in isolated unsealed human erythrocyte membranes the drug induced a disordering effect on the acyl chains of the membrane lipid bilayer; c) in scanning electron microscopy studies on human erythrocytes it was observed that the drug induced morphological changes different from their normal biconcave shape.

Keywords: cell membrane; diclofenac; phospholipid bilayer

Introduction

This article describes the effects of the anti-inflammatory drug diclofenac 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 cell 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 exeption, 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 eukariotic plasma membranes present a high percentage of the phospholipids phosphatidylcholines and spingpomyelins in the outer monolayer whereas the inner one is generally richer in phosphatidylethanolamines, phosphatidylserines and phosphatidylinositols. In the course of *in vitro* system search for the toxicity screening of biologically relevant

[1] Department of Polymers, Faculty of Chemical Sciences, University of Concepción, Casilla 160-C, Concepción, Chile
Fax: (+56) 41 2204171; E-mail: msuwalsk@udec.cl
[2] Faculty of Biological Sciences, University of Concepción, Concepción, Chile
[3] Institute of Chemistry, Catholic University of Valparaíso, Valparaíso, Chile

chemicals, different models have been applied in order to examine their adverse effects. Intended to better understand the molecular mechanisms of the interaction of diclofenac with cell membranes we have utilized human erythrocytes and molecular models of cell membranes. Human erythrocytes were chosen because since they have only one membrane and no internal organelles it is an ideal cell system for studying the interactions of chemicals with biomembranes.[4] Aditionally, although less specialized than many other cell membranes, they carry on enough similar functions, such as active and passive transport and the production of ionic and electric gradients, in order to be considered representative of the plasma membrane in general. The molecular models consisted of bilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), where DMPC represent phospholipid classes located in the outer monolayer of cell membranes, particularly of the human erythrocyte, whereas DMPE represents those preferentially located sited in the inner monolayers.[3,5]

Diclofenac, (2-[(2,6-dichlorophenyl)-amino]benzeneacetic acid, Fig. 1) is a non-steroidal anti-inflammatory drug with good analgesic properties; however, it may cause side effects including gastrointestinal disorders when administered by oral route and cutaneous lesions by intramuscular injection.[6] It also has cytotoxic effects and induces apoptosis in various cultured cell lines. Both toxic and apoptotic effects of diclofenac might be involved in

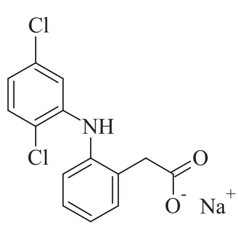

Figure 1.
Structural formula of diclofenac.

drug-induced Reye's syndrome, renal toxicity, hepatotoxicity, and pancytopenia.[7] Its effects on patient blood are significantly associated with aplastic and hemolytic anemia, thrombocytopenia and agranulocytosis.[8] The primary target of NSAIDs in relation to their direct cytotoxicity remains unknown.[9] One such target candidate is the cell membrane. It has been reported that NSAIDs interact with phospholipids, and that phospholipid liposomes reduce the direct cytotoxicity of in vivo NSAIDs.[10–12] On the other hand, it has been suggested that mitochondrial membrane permeability transition is a possible mechanism of diclofenac-induced apoptosis in hepatocytes.[7] It has been found that diclofenac induced membrane permeability transition in isolated rat liver mitochondria and in primary cultured hepatocytes.[13]

Materials and Methods

Reagents

Synthetic DMPC (lot 14OPC-241, MW 677.9), DMPE (lot 14OPE-58, MW 635.9), and sodium diclofenac (lot 0751896, MW 318.1) from Sigma (St. Louis, MO) were used without further purification.

X-Ray Diffraction Studies of DMPC and DMPE Multilayers

The capacity of diclofenac to perturb the structures of DMPC and DMPE multilayers was evaluated by X-ray diffraction. About 2 mg of each phospholipid was mixed in Eppendorf tubes with 200 μl of (a) distilled water and (b) aqueous solutions of diclofenac in a range of concentrations (0.01 mM to 2.0 mM). The specimens were incubated for 30 min at 37 °C and 60 °C with DMPC and DMPE, respectively and centrifuged for 10 min at 2500 rpm. The samples were then transferred to 1.5 mm dia special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany) and X-ray diffracted. 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 were obtained in an MBraun PSD-50M linear position-sensitive detector system (Garching, Germany) and no correction factors were applied. The experiments were performed at 18°C 1°C, which is below the main phase transition temperature of both DMPC (24.3°C) and DMPE (50.2°C).[14–15] 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.

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

The influence of diclofenac on the physical properties of DMPC LUV and IUM 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. Their steady-state fluorescence anisotropy measurements were used to investigate the structural properties of DMPC LUV and IUM 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 high excitation sensitivity 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 by means of the general polarization (GP) concept.[16] DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspensions (final lipid concentration 0.4 mM) through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp., MA, USA) under nitrogen pressure at 10°C above the lipid phase transition temperature. IUM were prepared by lysis, according to Dodge et al.[17] Erythrocytes were separated from heparinized venous blood samples obtained from normal casual donors by centrifugation and washing procedures. DPH and laurdan were incorporated into LUV and IUM by addition of 2 μl/mL aliquots of 0.5 mM solutions of the probe in dimethylformamide and ethanol, respectively, in order to obtain final analytical concentrations of 1×10^{-3} mM, and incubated them at 37°C for 45 min. Fluorescence spectra and anisotropy measurements were performed in a phase shift and modulation K_2 steady-state and time resolved spectrofluorometer (ISS, Inc., Champaign, IL, USA) interfaced to computer. Software from ISS was used for both data collection and analysis. LUV suspensions measurements were carried out at 18°C and 37°C, and IUM measurements were made at 37°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., Inc.) 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: $r = (I_{||} - I_{\perp}) / (I_{||} + 2I_{\perp})$, where $I_{||}$ and $I_{\perp}$ are the corresponding parallel and perpendicular emission fluorescence intensities with respect to the vertically polarized excitation light.[18] 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 both gel and liquid crystalline phases, respectively.[19] Diclofenac was incorporated in LUV and IUM suspensions by addition of adequate (10 mM) aliquots of a concentrated diclofenac in order to obtain the different concentrations used in this work. Samples thus prepared were then incubated at 37°C, for ca. 15 min; LUV were measured at 18°C and 37°C; at 18°C because the X-ray experiments were performed at about this temperature, and at 37°C because that is the normal temperature at which erythrocytes circulate in humans. Blank subtraction was performed in all measurements using unlabeled samples without probes. Data presented in Figures 3, 4 and 5 represent mean values and standard error of ten measurements in two independent samples. Unpaired Student's t-test was used for statistical calculations.

Scanning Electron Microscope (SEM) Studies of Human Erythrocytes

Blood was obtained from healthy human male donor not receiving any pharmacological treatment. Blood samples (0.1 ml) were obtained by puncture of the ear lobule and received in an Eppendorff tube containing 10 μl of heparin (5000 UI/ml) in 0.9 ml of saline solution (NaCl 0.9%, pH 7.4). The sample was centrifuged (1000 rpm × 10 min) and the supernatant was discarded and replaced by the same volume of saline solution; the whole process was repeated three times. The sedimentary red blood cells were suspended in 0.9 mL of saline solution and fractions of this stock of red blood cells suspension (RBCS) and saline solution were placed in Eppendorf tubes to prepare (a) the control, by mixing 100 μl of saline solution plus 100 μl RBCS, and (b) a range of concentrations of diclofenac (0.01 mM - 2mM) by mixing 100 μl of RBCS with 100 μl of adequate diclofenac stock solutions in saline solution. All samples were then incubated for one hour at 37°C. After the incubation, samples were centrifuged (1000 rpmx10 min) and the supernatant was discarded. Then, they were fixed overnight at 4°C by adding 500 μl of 2.5% glutaraldehyde to each one. The fixed samples were washed with distilled water, placed over Al glass cover stubs, air dried at 37°C for 30 min to 1 h, and gold-coated for 3 min at 10^{-1} Torr in a sputter device (Edwards S150, Sussex, England). Resulting specimens were examined in a Jeol SEM (JSM 6380 LB, Japan).

Results

X-Ray Diffraction Studies of DMPC and DMPE Multilayers

Fig. 2A exhibits the results obtained by incubating DMPC with water and diclofenac. As expected, water altered the DMPC structure: its bilayer repeat (bilayer width plus the width of the water layer between bilayers) increased from about 55 Å in its dry crystalline form[20] to 64.5 Å when immersed in water and its low-angle reflections, 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, which corresponds to the average distance between fully extended acyl chains organized with rotational disorder in hexagonal packing.[20] These results were indicative of the fluid state reached by DMPC bilayers. Fig. 2A discloses that after exposure to 0.1 mM diclofenac there was a weakening of the low- and wide-angle lipid reflection intensities (indicated as (a) and (b) in the figure, respectively); addition of diclofenac in increasing concentrations caused a monotonically decrease in the phospholipid reflection intensities, until they practically disappeared at 2 mM. From these results, it can be concluded that diclofenac produced a significant structural perturbation of DMPC bilayers. Fig. 2B

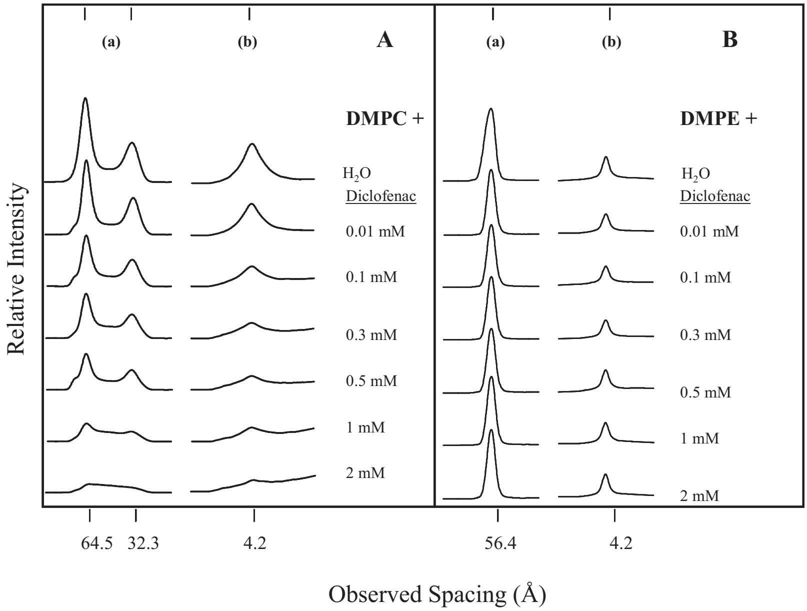

Figure 2.
Microdensitograms from X-ray diffraction patterns of (A) dimyristoylphosphatidylcholine (DMPC) and (B) dimyristoylphosphatidylethanolamine (DMPE) in water and aqueous solutions of sodium diclofenac; (a) low-angle and (b) wide-angle reflections.

shows the results of the X-ray diffraction analysis of DMPE bilayers incubated with water and diclofenac. As reported elsewhere, water did not significantly affect the bilayer structure of DMPE.[20] Fig. 2B also shows that increasing concentrations of diclofenac did not cause a weakening in DMPE reflection intensities, all of which

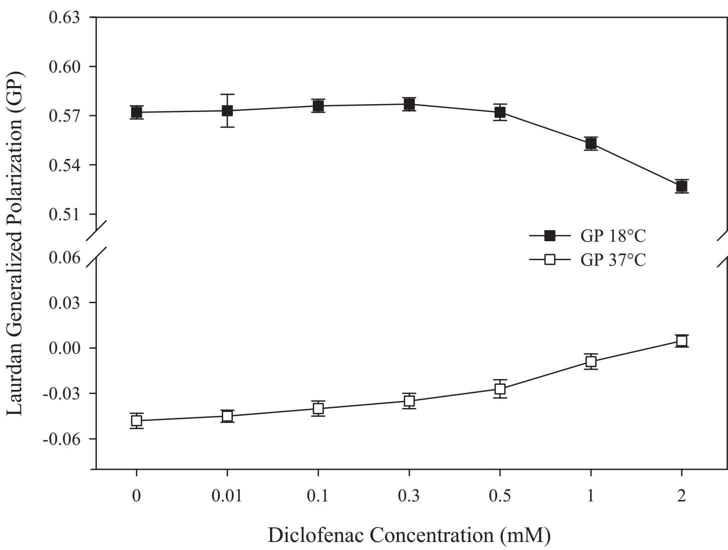

Figure 3.
Effects of sodium diclofenac on the generalized polarization (GP) of laurdan embedded in DMPC large unilamellar vesicles (LUV) at 18°C and 37°C.

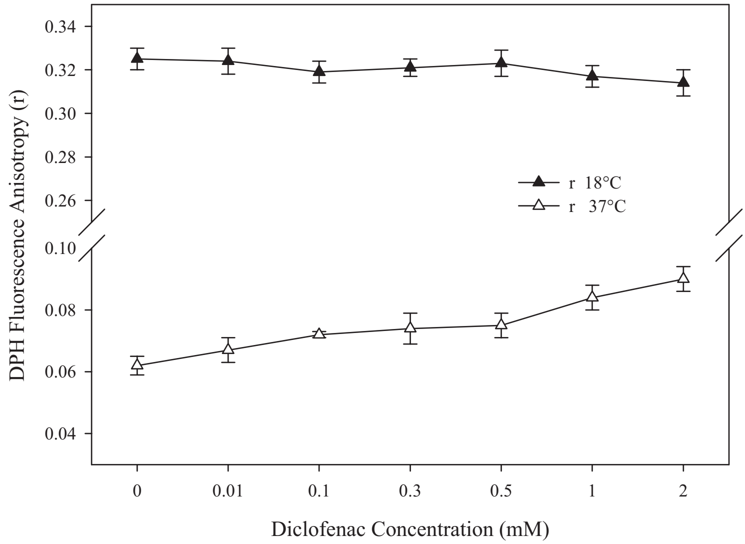

Figure 4.
Effects of sodium diclofenac on the anisotropy of DPH embedded in DMPC large unilamellar vesicles (LUV) at 18°C and 37°C.

still remained practically unaffected at the highest assayed diclofenac concentration. From these results, it can be concluded that diclofenac only induced structural perturbations to DMPC bilayers.

Fluorescence Measurements of Large Unilamellar Vesicles (LUV)
The concentration-dependent effect of diclofenac on DMPC LUV was explored at two different depths of the lipid bilayer:

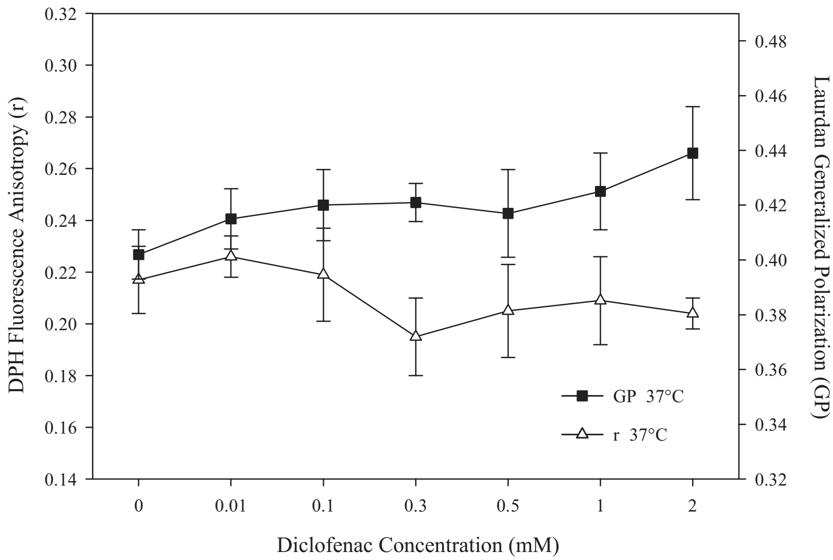

Figure 5.
Effects of sodium diclofenac on the anisotropy (r) of DPH and on the general polarization (GP) of laurdan embedded in isolated unsealed human erythrocyte membranes (IUM) at 37°C.

at the hydrophilic/hydrophobic 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). Fig. 3 shows that the incorporation of diclofenac to DMPC LUV in increasing concentrations induced a mild decrease of the GP values at 18°C and a mild increase at 37°C. Similar measurements of DPH fluorescence anisotropy (r) (Fig. 4) showed that while at 18°C remained practically constant, there was a mild increase at 37°C, implying a mild ordering of DMPC acyl chains. It should be taken into account that at 37°C this lipid is in a much more fluid state than at 18°C.

Fluorescence Measurements of Isolated Unsealed Human Erythrocyte Membranes (IUM)

The concentration-dependent effect of diclofenac on IUM was explored at the erythrocyte membrane lipid bilayer. As shown in Fig. 5, diclofenac incorporation at increasing concentrations up to 2 mM only

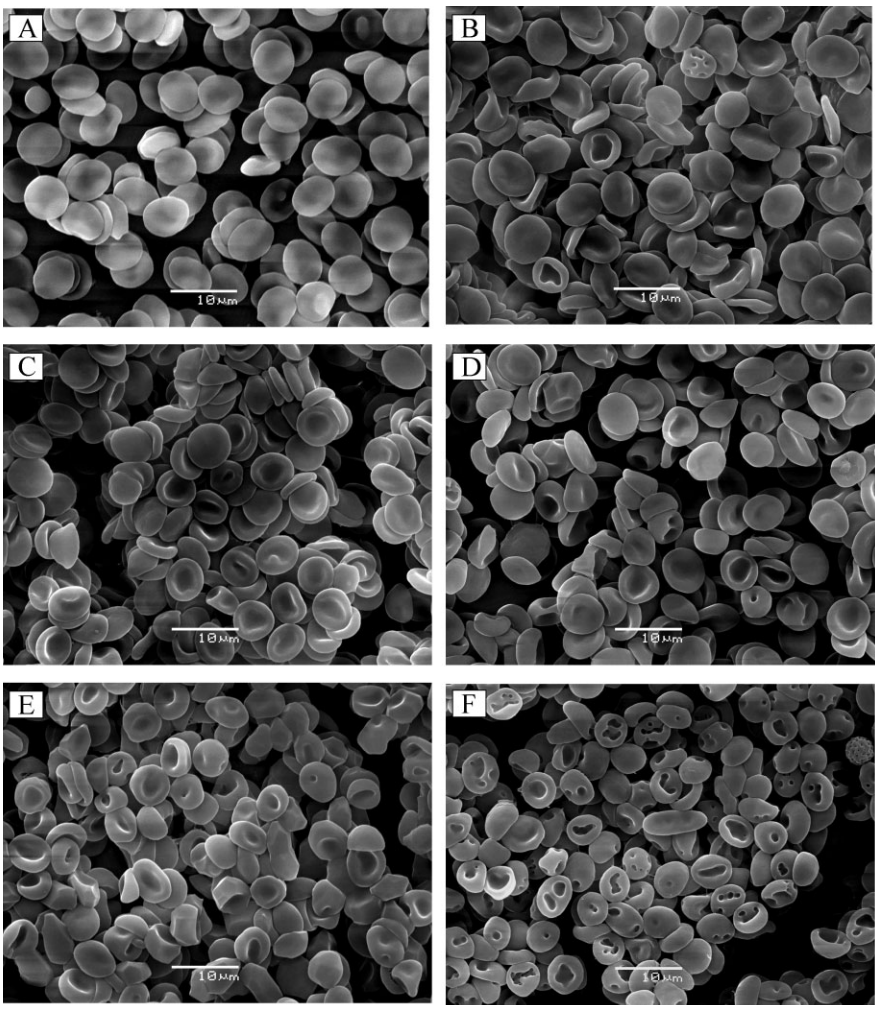

Figure 6.
Effects of sodium diclofenac on the morphology of human erythrocytes. SEM images of (A) untreated erythrocytes; incubated with (B) 0.01 mM; (C) 0.1 mM; (D) 0.3 mM; (E) 0.5 mM, and (F) 2 mM sodium diclofenac.

induced a mild increase of the laurdan GP and a mild decrease of DPH anisotropy up to 0.3 mM. These results imply a moderate rigidifyng of the bilayer polar groups organization, and a moderate disorder of the hydrophobic acyl chains.

Scanning Electron Microscope (SEM) Studies of Human Erythrocytes

SEM examinations of human erythrocytes incubated with diclofenac in the range of 0.01 mM-2 mM indicated that the drug induced morphological changes different from the normal biconcave shape of most red blood cells (Fig. 6A). With 0.01 mM diclofenac (Fig. 6B), about a third of the cells changed their discoid normal shape into stomatocytes (a cup-shaped form with evagination of one surface and a deep invagination of the opposite face), and another third into knizocytes (red blood cells with two or three concavities due to indentations in the cell membrane); with 0.1 mM (Fig. 6C) the majority of the cells were stomatocytes, with 0.3 mM (Fig. 6D) the majority of the cells were knizocytes, with 0.5 mM (Fig. 6E) there were many knizocytes and spherostomatocytes (cells with a visible change towards spheroid morphology with lightly or minor cupped profiles), and with 2 mM (Fig. 6F) the majority of the cells were knizocytes.

Conclusion

Studies on the effects of diclofenac on cell membranes are not too frequent, but a few of them have indicated that the drug produced some functional effects. Thus, Uyemura et al.[21] reported that diclofenac induced membrane permeability transition to rat renal mitochondrial, and uncoupled oxidative phosphorylation,[22] increased the fluidity of mouse splenocyte membrane,[23] caused cell death in human hepatocytes through mitochondrial permeabilization,[24] and activates K^+ channels exerting its effect at both inner and outer sides of rat cerebellar granule cells.[25] However, it becomes amazing the lack of information on the effects induced by diclofenac to human erythrocytes. One of the few reports indicates that diclofenac at the therapeutic and higher concentrations exerts in vitro an inhibition on H_2O_2 forced erythrocytic membrane lipid peroxidation as well as increased hemolysis.[26] The present study presents the following evidence that diclofenac affects human erythrocytes and molecular models of its cell membrane: X-ray diffraction experiments were performed on bilayers made up of DMPC and DMPE, classes of the major phospholipids present in the outer and inner erythrocyte membrane, respectively. Results showed that diclofenac interacted practically only with DMPC, affecting both its polar head and acyl chain regions. DMPC and DMPE differ only in their terminal amino groups, being these $^+N(CH_3)_3$ in DMPC and $^+NH_3$ in DMPE. Moreover, both molecular conformations are very similar in their dry crystalline phases[20] 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 resulting width increase. This phenomenon allows the incorporation of diclofenac into DMPC bilayers with the resulting disruption of DMPC bilayer structure. Similar results were observed on unilamellar liposomes and floating Langmuir monolayers of soya phosphatidylcholine.[27] Given the amphiphilic nature of sodium diclofenac it is very likely that it locates into DMPC bilayer in such a way that its negatively charged carboxyl group electrostatically interacts with the positively charged DMPC terminal $^+N(CH_3)_3$ group, while the apolar ring sits in the neighborhood of the hydrophobic acyl region of DMPC. A somewhat similar conclusion has been suggested by Lichtenberger,[16] and reported on the basis of ^{31}P NMR spectra of phosphatidylcholine liposomes.[28] As diclofenac is negatively charged its location in the polar region of DMPC modifies the electrostatic interactions between the lipid phosphate and terminal amino groups disrupting its bilayer structure. However, other explanations cannot be disregarded. One of them considers the hydration, which plays an important role in the stability of phospholipids bilayers. In both gel and liquid crystalline phases water molecules are bound to DMPC head group. The molecules are oriented and form a hydration shell through hydrogen bonding with the polar groups. In the presence of diclofenac the amount of water molecules, their orientation and hydrogen bonds can be affected. Thus, the presence of diclofenac negatively charged carboxyl group located near the phosphate region can compete for the formation of hydrogen bonds with the water molecules of the hydration shell. The weakening of the water binding to the phosphate region would modify DMPC affinity for water and consequently the packing of its bilayer. On the other hand, DMPE is not significantly affected by diclofenac, the explanation lying in that the molecules pack tighter than those of DMPC due to their smaller polar groups and higher effective charge, resulting in a very stable bilayer system held by electrostatic interactions and hydrogen bonds.[20]

The toxic effect due to diclofenac treatment was also observed on erythrocytes in the same concentration range. In fact, scanning electron microscopy (SEM) observations of human erythrocytes indicated that they underwent a morphological alteration as their discoid normal shape changed with increasing diclofenac concentrations. According to the bilayer-couple hypothesis,[29] shape changes are induced in red cells due to the insertion of foreign species in either the outer or the inner monolayer of the erythrocyte membrane. Thus, spiculated shapes (echinocytes) are observed in the first case while cup shapes (stomatocytes) are produced in the second due to the differential expansion of the corresponding monolayer. Given the extent of the interaction of diclofenac with DMPC, the lipid class preferentially located in the outer monolayer of the erythrocyte membrane, echinocytes, were expected from the erythrocytes incubated with the drug instead of the mostly observed stomatocytosis. The explanation for this discrepancy could be based on the lipid scrambling mechanism proposed by Schrier et al.[30] According to it, some cationic amphipaths produce a rapid scrambling of the erythrocyte bilayer with phosphatidylcholines (PC) and sphyngomyelins (SM) moving inward while phosphatidylethanolamines (PE) moves outward along with phosphatidylserines (PS). Thus, the interaction of diclofenac 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 Schrier et al.[30] An alternative explanation might lie in that diclofenac interacted with proteins located in the inner monolayer of the erythrocyte membrane. On the other hand, the concentration-dependent effect of diclofenac on isolated unsealed human erythrocyte membrane (IUM) was explored by fluorescence spectroscopy at two different depths: at the hydrophilic/hydrophobic level and in the deep hydrophobic core of the membrane lipid bilayer. The incorporation of diclofenac induced a mild increase of the generalized polarization, result that can be rationalized as a moderate decrease in the molecular dynamics and/or water content at the glycerol backbone level of the polar head groups, whereas the moderate decrease of the anisotropy up to 0.3 mM implies a moderate disorder of the acyl chain packing order at the bilayer hydrophobic core. Interestingly, the corresponding results of both probes embedded in DMPC LUV clearly show that in liquid crystalline phase at 37 °C diclofenac exerts a rigidifying effect on DMPC bilayer. The difference in DPH anisotropy behavior in both systems could be ascribed to the presence of lipid-protein interfaces in IUM.

Amazingly, the therapeutic range for plasma diclofenac concentrations and the relationship of plasma concentration to clinical response and toxicity have not been clearly established. Nevertheless, the experimental findings are certainly of interest as they indicate that a diclofenac concentration as low as 10 μM affects the human erythrocyte shape. It must be considered that alteration of the normal biconcave shape of red blood cells increases their resistance to entry into capillaries,[31] which could contribute to decreased blood flow, loss of oxygen, and tissue damage through microvascular occlusion.[32]

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