EXERCISE IV.1
TOXICITY SCREENING OF THERAPEUTIC DRUGS

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INTRODUCTION

In the course of in vitro systems search for the toxicity screening of therapeutic drugs (TDs), different cellular models have been applied to examine their adverse effects in isolated organs. This article describes a simple method to determine effects of TDs 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 responsible 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 phosphatidylethanolamines, phosphatidylserines, and phosphatidylinositols. However, the existence of asymmetric plasma membranes is less certain in bacteria than in eukaryotes. Studies of the phospholipid distribution of a gram-positive bacteria revealed that the outer monolayer is rich in phosphatidylglycerols, the inner one in phosphatidylinositols, while cardiolipins are symmetrically distributed between both monolayers. Studies on gram-negative bacteria such as *Escherichia coli* have detected phosphatidylethanolamines in the outer membrane, whereas the cytoplasmic membrane has been reported to be rich in phosphatidylglycerols and cardiolipins.

With the aim to better understand the molecular mechanisms of the interaction of TDs with cell membranes, we utilize a well-established model consisting of intact human

erythrocytes and molecular models of its membrane. The latter consist of bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes respectively located in the outer and inner monolayers of erythrocytes and other cell membranes. Erythrocytes were chosen because although less specialized than many other cell membranes, they carry on enough functions in common with them such as active and passive transport, and the production of ionic and electric gradients, to be considered representative of the plasma membrane in general. The capacity of TDs to interact with the erythrocyte membrane can be determined by scanning electron microscopy (SEM), whereas the interaction with the bilayer structures of DMPC and DMPE can be defined 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 [4–5], antiarrhythmic [6], and anticancer drugs [7–8].

MATERIAL AND METHODS

X-ray diffraction analysis of phospholipid bilayers

The capacity of TDs to perturb the structures of DMPC and DMPE bilayers is to be determined by X-ray diffraction. For this purpose, about 1 mg of each phospholipid (Sigma or Polar Avanti, USA) is introduced into 2-mm-diameter special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany), which are then filled with about 200 μL of (a) distilled water and (b) aqueous solutions of the TD in a range of concentrations. The experiments must be performed at 17 ± 2°C, which is below the main phase transition temperature of both DMPC and DMPE.

SEM studies on human erythrocytes

Blood samples can be taken from clinically healthy adult donors by puncture of the ear lobe disinfected with ethanol and aspiration into a tuberculin syringe without a needle containing 50 units/mL heparin in saline solution (0.9 % NaCl). Centrifuge the red blood cells for 10 min at 1000 rpm, wash twice in saline solution, resuspend in saline solutions containing the TD in adequate concentrations, and incubate for 1 h at 37 ºC. Control are cells resuspended in saline solution without TD. Fix the specimens overnight at 5 ºC by adding one drop of each sample to plastic tubes containing 1 mL of 2.5 % glutaraldehyde, wash twice in distilled water, place them on siliconized Al stubs

and air-dry at 37 ºC for 30 min. Gold-coat the Al stubs for 3 min at 13.3 pascal in a sputter device and examine the samples in an SEM.

DESCRIPTION OF RESULTS

In order to understand the possible results to be obtained, a description of the experimental results observed with the local anesthetic bupivacaine is presented.

X-ray diffraction studies

Figure 1A shows a comparison of the diffraction patterns of DMPC alone immersed in excess water and those of DMPC incubated with bupivacaine in the range of 0.1 mM up to 15 mM. The reflections labeled (a) correspond to the 64 Å distance between DMPC polar groups (see Fig. 2a), whereas the strong reflection of 4.2 Å labeled (b) corresponds to the average distance between DMPC fully extended acyl chains organized with rotational disorder in hexagonal packing. It is noticeable that addition of 0.1 mM bupivacaine caused only a very slight decrease in the phospholipid reflection intensities, but 1 mM induced a marked decrease of the (a) intensities, whereas the intensity of 4.2 Å reflection was essentially unchanged. However, 8 mM bupivacaine induced a marked decrease of the 4.2 Å reflection intensity and the complete disappearance of the (a) reflections, which were replaced by a diffuse halo. This pattern remained practically unchanged after exposure to 15 mM bupivacaine. These results imply that bupivacaine 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 bupivacaine with DMPE. The perturbing effect of this compound upon the structure of DMPE bilayers was practically negligible even at a 23 mM concentration. 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. Moreover, both molecular conformations are very similar with the hydrocarbon chains mostly parallel and extended, and the polar groups lying perpendicular to them (Fig. 2b). However, the hydration of DMPC results in water filling the highly polar interbilayer spaces, a phenomenon that allows the incorporation of bupivacaine into DMPC bilayers, producing its structural perturbation and almost complete destruction at a 8 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 significantly affected by water nor by a number of compounds.

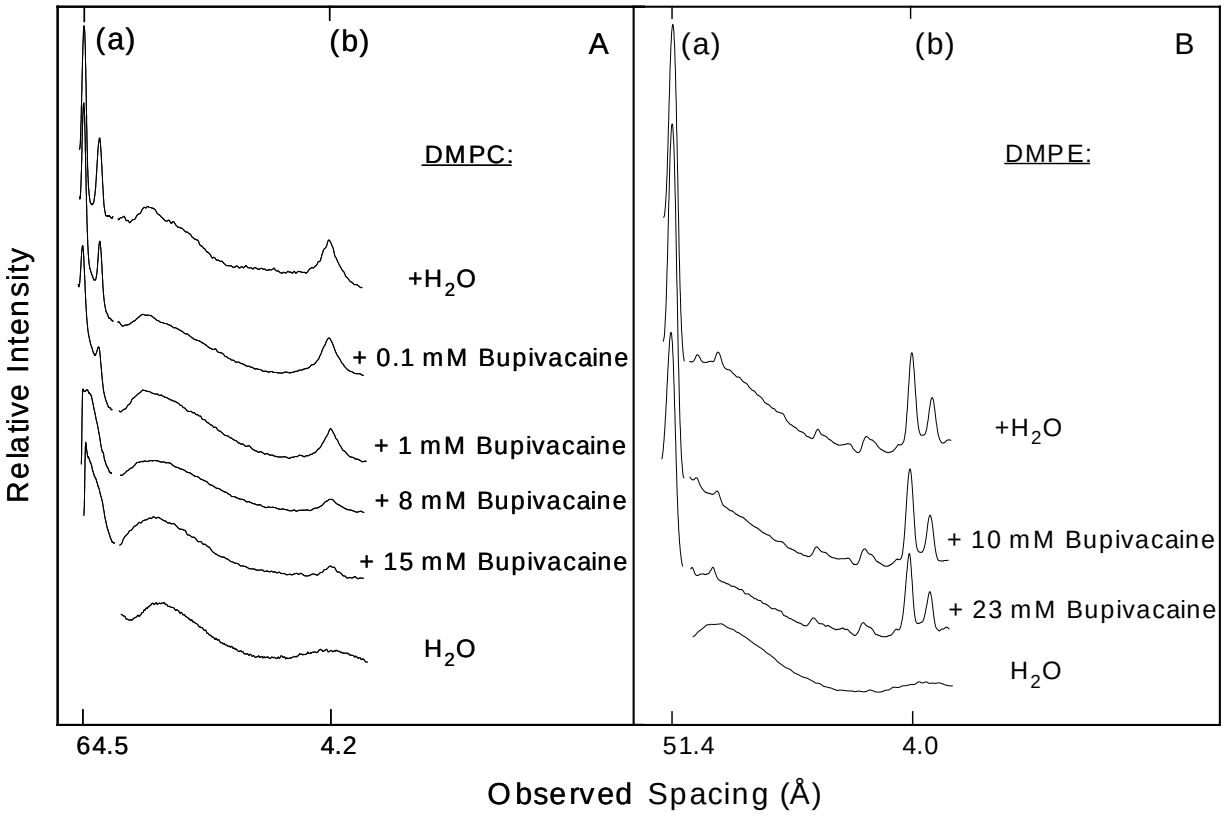


Fig. 1 X-ray diffraction diagrams of DMPC (A) and DMPE (B) in water and aqueous solutions of bupivacaine; (a) reflections arising from the polar group and (b) from the acyl chain regions.

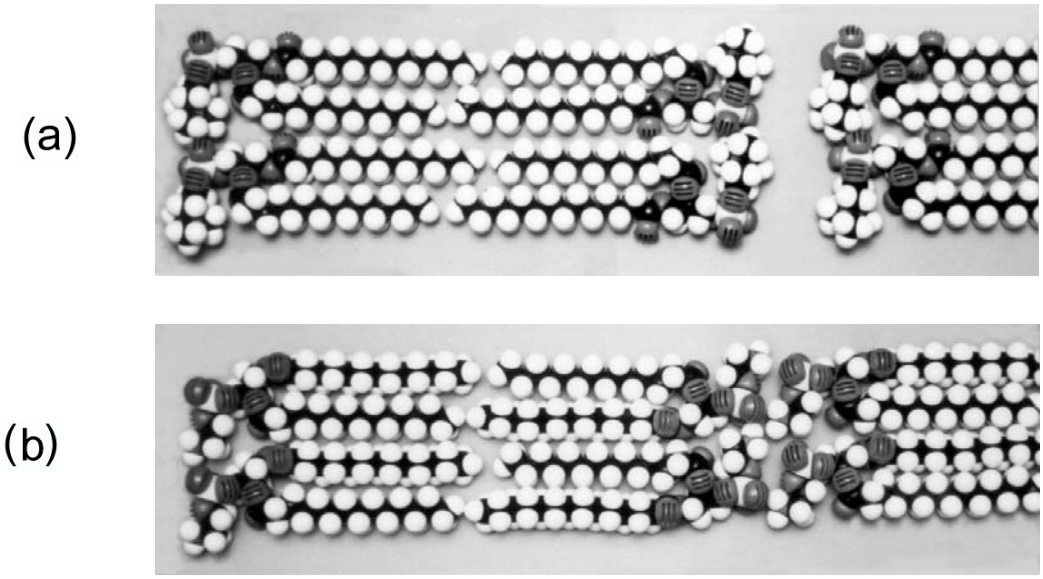
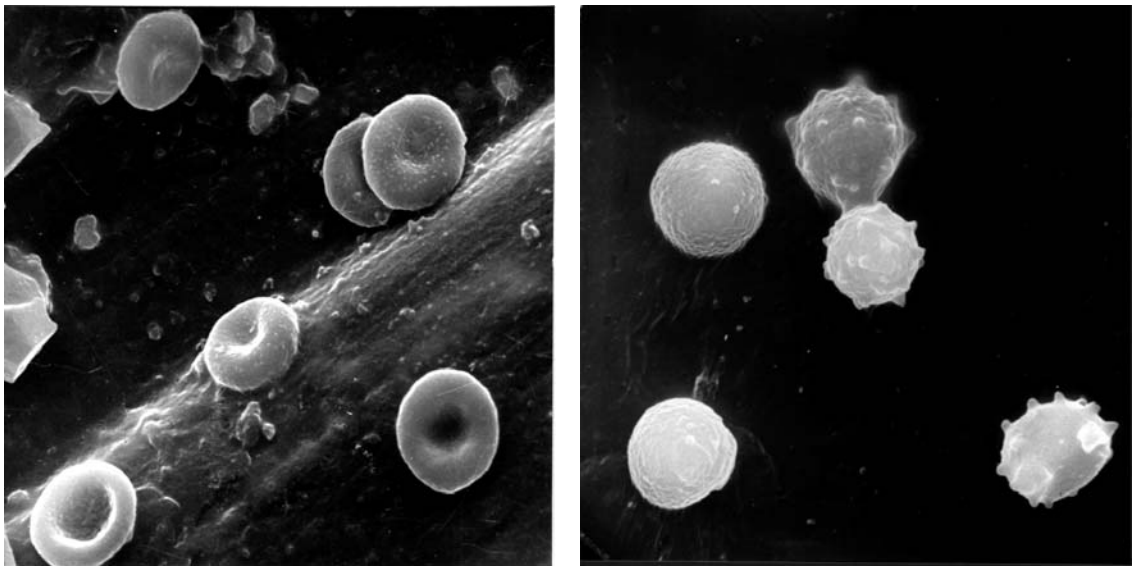


Fig. 2 Two-dimensional packing arrangements of (a) DMPC and (b) DMPE bilayers.

SEM studies on human erythrocytes

Human red blood cells were incubated with 3 mM bupivacaine. The phase contrast and SEM observations indicated that bupivacaine induced a significant change in the shape



A B

Fig. 3 Effect of bupivacaine on the morphology of human erythrocytes. SEM images of (A) control (2500X) and (B) erythrocytes incubated with 3 mM bupivacaine (2400X).

of the erythrocytes. In fact, the erythrocytes underwent a morphological alteration as they changed their discoid shape (Fig. 3A) to spheroechinocytes (Fig. 3B). According to the bilayer couple hypothesis [9], the shape changes induced in erythrocytes by foreign molecules are due to differential expansion of their two monolayers. Thus, spiculated 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 bupivacaine produced spheroechinocytes would indicate that the anesthetic was located in the outer moiety of the red cell membrane.

In conclusion, the experimental results indicate that bupivacaine interacts with the human erythrocyte membrane, the anesthetic being located in the outer moiety of the red cell membrane. The X-ray experiments, performed on bilayers made up of classes of the major phospholipids present in either the outer and inner sides of the erythrocyte membrane, confirmed this result. In fact, they showed that 1 mM bupivacaine slightly disordered the polar head region of DMPC (major class of lipid present in the outer monolayer of the erythrocyte membrane) and 8 mM completely perturbed it, whereas 23 mM bupivacaine produced negligible effects on DMPE (which preferentially locates in the erythrocyte inner monolayer).

REFERENCES

[1] Membrane Physiology, T.E. Andreoli, J. F. Hoffman, D.D. Fanestil, eds., Plenum, 1980.

[2] In Search of a New Biomembrane Model, O.G. Mouritsen, O.S. Anderen, eds., Munksgaard, 1998

[3] J.M. Boon, B.D. Smith, Chemical Control of Phospholipid Distribution Across Bilayer Membranes, Med. Res. Rev. 22 (2002), 251-281.

[4] M. Suwalsky, C. Schneider, F. Villena, B. Norris, H. Cárdenas, F. Cuevas, C.P. Sotomayor, Structural Effects of the Local Anesthetic Bupivacaine Hydrochloride on the Human Erythrocyte Membrane and Molecular Models, Blood Cells, Mol. Dis. (2002), 29, 14-23.

[5] M. Suwalsky, C. Schneider, F. Villena, B. Norris, H. Cárdenas, F. Cuevas, C.P. Sotomayor, Effects of the Local Anesthetic Benzocaine on the Human Erythrocyte Membrane and Molecular Models, Biophys. Chem. (2004), 109, 189-199.

[6] M. Suwalsky, I. Sánchez, M. Bagnara, C.P. Sotomayor, Interaction of Antiarrhythmic Drugs with Model Membranes, Biochim. Biophys. Acta (1994) 1195, 189-196.

[7] M. Suwalsky, P. Hernández, F. Villena, F. Aguilar, C.P. Sotomayor, The Anticancer Drug Adriamycin Interacts with the Human Erythrocyte Membrane, Z. Naturforsch. (1999), 54c, 271-277.

[8] M. Suwalsky, P. Hernández, F. Villena, C.P. Sotomayor, The Anticancer Drug Chlorambucil Interacts with the Human Erythrocyte Membrane and Model Phospholipid Bilayers, Z. Naturforsch. (1999) 54c, 1089-1095.

[9] M.P. Sheetz, S.J. Singer. Biological Membranes as Bilayer Couples. A Molecular Mechanism of Drug-Erythrocyte Induced Interactions. Proc. Natl. Acad. Sci. USA (1974) 71, 4457-4461.

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