

# The Local Anesthetic Proparacaine Modifies Sodium Transport in Toad Skin and Perturbs the Structures of Model and Cell Membranes

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## Local Anesthetic, Proparacaine, Erythrocyte Membrane

Experimental results indicate a significant decrease in the potential difference (PD) and in the short-circuit current (I<sub>sc</sub>) after the application of proparacaine to isolated toad skin, which may reflect an inhibition of the active transport of ions. This finding was explained on the basis of the results obtained from membrane models incubated with proparacaine. These consisted of human erythrocytes, isolated unsealed human erythrocyte membranes (IUM), phospholipid multilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representatives of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively, and in large unilamellar vesicles (LUV) of DMPC. X-ray diffraction showed that proparacaine interaction with DMPC and DMPE bilayers perturbed both structures, especially DMPC. This result, confirmed by fluorescence spectroscopy of DMPC LUV at 18 °C, demonstrated that the local anesthetic (LA) could interact with the lipid moiety of cell membranes. However, effects observed by scanning electron microscopy (SEM) of human erythrocytes and by fluorescence spectroscopy of IUM might also imply proparacaine-protein interactions. Thus, the LA may alter epithelial sodium channels through interaction with the lipid matrix and with channel protein residues.

## Introduction

Local anesthetics (LA) belong to a relatively large number of compounds which induce voltage and/or frequency-dependent block of sodium (Scheuer, 1999; Baker and Wood, 2001), potassium (González *et al.*, 2001; Olschewski *et al.*, 1999) or calcium channels (Xu *et al.*, 1993). As the anesthetic action progressively develops in a nerve, the threshold for electrical excitability gradually increases, the rate of rise of the action potential declines, impulse conduction slows, and the safety factor for conduction decreases. These factors decrease the probability of propagation of the ac-

tion potential and the nerve conduction fails (Catterall and Mackie, 2001). The two compounds most frequently used in ophthalmology for anesthesia of the cornea and conjunctiva are proparacaine and tetracaine. These LA can retard corneal healing (Catterall and Mackie, 2001) and inhibit the voltage-gated sodium current of the nerve membrane (Sugiyama and Muteki, 1994). Voltage-sensitive Na<sup>+</sup> channels are chief targets of anesthetic drugs, where S6 is apparently the physical activation gate (Marban *et al.*, 1998). It is not known whether anesthetic concentrations of proparacaine depress epithelial sodium transport, since the Na<sup>+</sup> channels found in the apical membrane of epithelia (ENaC) belong to another family with a structure different from voltage-gated channels (Benos and Stanton, 1999). In order to examine the effect of proparacaine on ENaC, experiments were performed on isolated amphibian skin epithelium, a known biological model for sodium-absorbing membranes (Brodin and Nielsen, 2000; Bernick and Stiffler, 2000). Na<sup>+</sup> enters the

**Abbreviations:** LA, local anesthetics; IUM, isolated unsealed human erythrocyte membrane; LUV, large unilamellar vesicles; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; PD, potential difference; I<sub>sc</sub>, short-circuit current; r, anisotropy; GP, general polarization; DPH, 1,6-diphenyl-1,3,5-hexatriene; laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; SEM, scanning electron microscopy; ENaC, epithelial sodium channels.

cell via apical, mainly amiloride-sensitive  $\text{Na}^+$  channels (Flonta *et al.*, 1998) and is extruded across the basolateral membrane by the  $\text{Na}^+/\text{K}^+$ -ATPase. Many of the mechanisms involved in transepithelial  $\text{Na}^+$  transport have been described using this preparation (Brodin *et al.*, 1996; Rytved and Nielsen, 1999). The short-circuit current ( $\text{I}_{\text{sc}}$ ), which is the amount of current necessary to bring the potential difference (PD) across the skin to zero, measures the net transepithelial  $\text{Na}^+$  absorption (Nielsen, 1997).

Although the precise molecular mechanisms of LA actions are not yet well understood, their lipophilicity make lipid-rich membranes sensitive target sites for their interaction with living organisms. It has been reported that they bind to a variety of cell membranes and induce functional perturbation of membrane proteins such as calmodulin,  $\text{Na}^+$  and  $\text{K}^+$  channels, acetylcholine receptors, ATPases, cytochrome oxidase and G proteins (Butterworth and Strichartz, 1990; de Paula and Schreier, 1996). It has been suggested that changes in the molecular organization of membranes, ranging from an increase in fluidity to lateral phase separation and alteration of lipid-protein interaction (melting of the lipid annulus) are involved in the mechanism of anesthesia (de Paula and Schreier, 1996). This is consistent with the hypothesis that alterations in the organization of lipid bilayers are likely to constitute a general mechanism for the modulation of membrane protein functions (Lundbaek *et al.*, 1996). Indeed, many reports confirm the interaction of LA with phospholipid bilayers (de Paula and Schreier, 1996; Kaneshina *et al.*, 1997; Kuroda *et al.*, 2000; Pinto *et al.*, 2000; Suwalsky *et al.*, 2001). For these reasons we thought it of interest to examine the binding affinity of proparacaine with cell membranes and its perturbing effects upon the phospholipid bilayer structures. With these aims we used a) human erythrocytes, which were investigated by phase contrast and scanning electron microscopy (SEM); b) isolated unsealed human erythrocyte membranes (IUM), studied by fluorescence spectroscopy; c) multibilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), which represent phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively, which were analyzed by X-ray dif-

fraction, and d) large unilamellar vesicles (LUV) of DMPC, examined by fluorescence spectroscopy. These systems have also been used to determine the interaction with, and perturbing effects on membranes by antiarrhythmic (Suwalsky *et al.*, 1994) and anticancer drugs (Suwalsky *et al.*, 2000), the LA dibucaine (Suwalsky *et al.*, 2001a); pesticides (Suwalsky *et al.*, 2001b) and metallic ions (Suwalsky *et al.*, 2001c).

## Materials and Methods

### *Electrophysiological measurements with isolated toad skin*

*Pleurodema thaul* toads of either sex (10–25 g) collected in fresh water ponds were kept in tap water 24 h prior to use and fed on sow bugs (*Oniscus asellus*). The experiments, carried out at room temperature (18–22 °C) were performed on sections of abdominal skin dissected from pithed toads. Samples were mounted between two halves of an Ussing-type chamber: a circular area of 1  $\text{cm}^2$  was exposed to 3 ml Ringer's bathing solution on each side. The composition of the solution was (mm):  $\text{Na}^+$  114,  $\text{K}^+$  2.5,  $\text{Cl}^-$  117.5,  $\text{Ca}^{2+}$  2.0,  $\text{HCO}_3^-$  2.3 and glucose 11. The bathing medium was oxygenated with a model Elite Hagen aerator. The  $\text{I}_{\text{sc}}$  was monitored with non-polarizable Ag/AgCl electrodes placed at 15 mm distance from the epithelium and connected to a voltage-clamp circuit (G. Métraux Electronique, Crissier, Switzerland) set to keep the PD across the skin at zero mV. The PD was measured with calomel-agar electrodes at intervals of 2 min for 4 s. Both parameters were displayed on a 2-channel Cole-Parmer recorder. 30 min after steady readings had been obtained, proparacaine was applied in the solution bathing either the outer or the inner surface of the skin in the final concentrations specified in the text. Results are expressed as means  $\pm$  S. E. Student's paired *t* test was used for statistical analysis.

### *Scanning electron microscopy (SEM) studies on human erythrocytes*

*In vitro* interaction of proparacaine with erythrocytes was achieved by incubating human blood samples taken from healthy male adult donors not currently receiving treatment with any pharmacological agent. Blood samples were obtained after

puncture of the ear lobe disinfected with 70% ethanol by aspiration into plastic tuberculin syringes without needles, containing 50 Units/ml heparin in saline solution (0.9% NaCl). Red blood cells were centrifuged, washed twice in saline, resuspended in buffer (7.5 mM phosphate, 145 mM NaCl, 5 mM glucose, 1 mM MgSO<sub>4</sub>, pH 7.4) containing proparacaine at a final 3 mM concentration and incubated for 1 h at 37 °C. Controls were erythrocytes resuspended in incubation buffer without proparacaine. Red blood cells were then fixed overnight at 5 °C by adding one drop of each sample to plastic tubes containing 1 ml of 2.5% glutaraldehyde in saline, washed twice with saline, placed on siliconized Al stubs, air-dried at 37 °C for 30 min and gold coated for 3 min at 10<sup>-1</sup> mm Hg in a S 150 sputter device (Edwards S150, Sussex, England). Resulting specimens were examined in an Etec Autoscan SEM (Etec, Corp, Hayward, CA, USA).

*Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)*

The influence of proparacaine 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 of the dynamic properties at the level of the phospholipid glycerol backbone. The quantification of the laurdan fluorescence shift was effected using the general polarization (GP) concept (Parasassi and Gratton, 1995), which is related to the lipid polar head organization in the zone of the erythrocyte membrane and DMPC LUV.

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.* (1963). DMPC LUV suspended in water were prepared by 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 °C above the lipid phase transition temperature. 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 °C for 45 min. Fluorescence spectra and anisotropy measurements were performed on a Spex Fluorolog (Spex Industries Inc., Edison, N. J., USA) and in a phase shift and modulation Gregg-200 steady-state and time-resolved spectrofluorometer (ISS Inc., Champaign, IL, USA) respectively, both interfaced to computers. Software from ISS was used for data collection and analysis.

Measurements of LUV suspensions were made at 18 °C and 37 °C and measurements of IUM were made at 37 °C using 10 mm path-length square quartz cuvettes. Sample temperature was monitored by an external bath circulator (Cole-Parmer, Chicago, IL, USA) and controlled before and after each measurement using an Omega digital thermometer (Omega Engineering Inc., Stamford, 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. Laurdan fluorescence spectral shifts were quantitatively evaluated using the GP concept (see above) which is defined by the expression GP = (I<sub>b</sub> - I<sub>r</sub>) / (I<sub>b</sub> + I<sub>r</sub>), where I<sub>b</sub> and I<sub>r</sub> 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 (Parasassi *et al.*, 1990). Proparacaine was incorporated in IUM and LUV suspensions by addition of small aliquots of a concen-

trated solution and incubated at 18 °C or 37 °C, depending on the work temperature, for ca. 15 min. Blank subtraction was performed in all measurements using labeled samples without probes.

#### *X-ray diffraction studies of phospholipid multilayers*

Synthetic DMPC (lot 80H8371, A grade, MW 677.9) and DMPE (lot 13H83681, A grade, MW 635.9) and proparacaine hydrochloride (lot 70H05991, MW 330.9) from Sigma (MO, USA) were used without further purification. About 3.5 mg of each phospholipid were mixed in 2.0 mm dia glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany) with 200 µl of aqueous proparacaine solutions (concentration range 0.1 mM to 25 mM). They were X-ray diffracted 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 CuK $\alpha$  radiation from a Philips PW 1140 X-ray generator was used. The relative reflection intensities on film were measured by peak integration using a 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 ± 2 °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.

## Results

### *Electrophysiological measurements on the isolated toad skin*

The electrical response of the toad skin to proparacaine applied in the solution bathing either the outside or the inside surface was a decrease in Isc and PD. Figure 1 shows that a 1.8 mM concentration (inside surface) was followed by a rapid decline of the electrical parameters which reached a trough in 51.8 ± 3.4 min, an effect which may be interpreted as a decrease in transepithelial ion transport (Nielsen, 1997). The effect was concentration-dependent (Fig. 2A and B) with a concen-

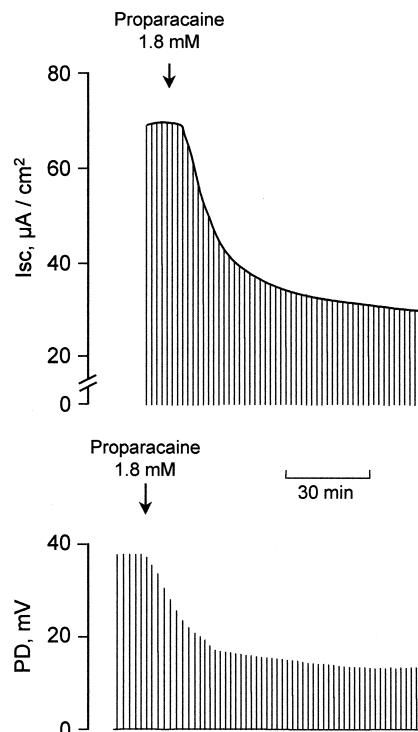


Fig. 1. Tracing representative of seven experimental runs showing the effect of proparacaine (1.8 mM, inside surface) on the bioelectric parameters of the isolated toad skin. Isc = short-circuit current; PD = potential difference.

tration for half-maximal response (EC<sub>50</sub>) of about 0.87 mM and was slightly although significantly greater (9.7 ± 0.8%, P < 0.02 and < 0.01 for Isc and PD respectively) when the anesthetic was applied to the inside surface. This effect is consistent with sodium channel blockade from the cytoplasmic mouth of the channel or with perturbation of membrane-bound enzyme activity. For both surfaces of the skin, the maximal (1.8 mM) concentration reduced the electric parameters by over 40%. Complete reversibility could only be achieved at low concentrations (< 0.6 mM); otherwise inhibition became partially irreversible in spite of wash-out. There was no significant change in resistance across the epithelium, indicating that membrane integrity was not disrupted at the maximal concentration used.

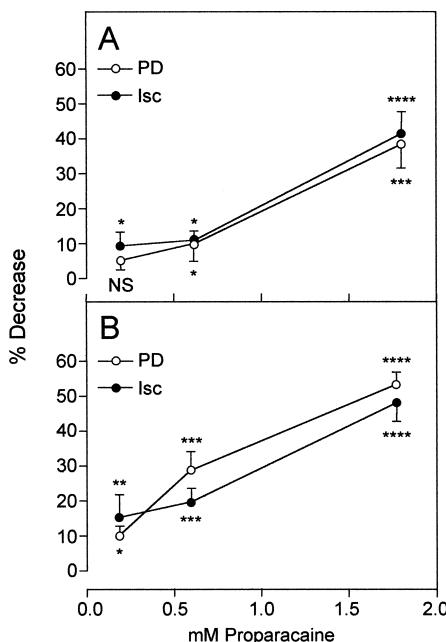


Fig. 2. Effects of proparacaine in increasing concentrations on the electric properties of the isolated toad skin. Potential difference (PD) and short-circuit current (Isc) values for untreated skins were  $28.5 \pm 3.0$  mV and  $45.8 \pm 4.0$   $\mu\text{A}/\text{cm}^2$  respectively. Results obtained in the presence of proparacaine (0.18, 0.60 and 1.80 mM) are expressed as the percentage decrease of these control values. Each point represents means  $\pm$  S.E.;  $n = 9$ . A) proparacaine applied to the outside surface; B) proparacaine applied to the inside surface. Significance by Student's paired *t* test: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ; NS = not significant.

#### Scanning electron microscopy (SEM) studies on human erythrocytes

SEM of red cells incubated with 3 mM proparacaine revealed abnormalities in their shapes. In contrast to the normal discoid erythrocyte profile (Fig. 3A) the proparacaine treated cells underwent stomatocytic shape changes, i.e., evagination of one surface and invagination of the opposite face (Fig. 3B). According to the bilayer couple hypothesis (Sheetz and Singer, 1974), the shape changes induced in erythrocytes by foreign molecules are due to differential expansion of their two monolayers. Thus, spiculated shapes (echinocytes) are formed 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 proparacaine induced the formation of stomatocytes would indi-

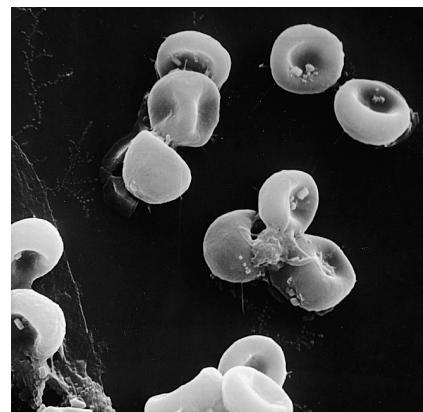
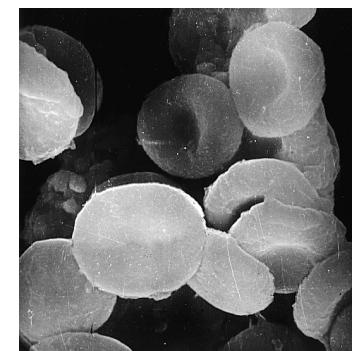


Fig. 3. Scanning electron microscope (SEM) images of human erythrocytes. (A) Control, 2900X; (B) incubated with 3 mM proparacaine, 2000X.

cate that the anesthetic was located in the inner moiety of the red cell membrane.

#### Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)

The interactions of proparacaine with IUM and DMPC LUV were determined at the acyl chain hydrophobic core and at the hydrophilic/hydrophobic interface regions of the phospholipid bilayers by evaluation of DPH steady state fluorescence anisotropy (*r*) and laurdan general polarization (GP), respectively. Table I shows that increasing concentrations of proparacaine (0 mM to 9.94 mM) decreased the *r* values of the erythrocyte membranes by an order of 10% and the GP values by about 39% at 37 °C. The results of the interaction of proparacaine with DMPC LUV at 37 °C and 18 °C are presented in Table II. As may be perceived, at 37 °C increasing concentrations of

Table I. Effect of proparacaine on the anisotropy (r) of DPH and the general polarization (GP) of laurdan embedded in isolated unsealed human erythrocyte membranes (IUM) at 37 °C.

Proparacaine (mM)	r (DPH)	GP (laurdan)
0	0.221 ± 0.001	0.234 ± 0.002
0.2	0.214 ± 0.002	0.231 ± 0.001
0.7	0.218 ± 0.002	0.241 ± 0.016
1.0	0.212 ± 0.002	0.224 ± 0.004
5.1	0.205 ± 0.002	0.185 ± 0.004
9.9	0.199 ± 0.002	0.143 ± 0.004

the anesthetic (0 to 6.06 mM) induced higher values of both parameters (r and PG) by an order of 67% and 212%, respectively. However, opposite results were observed at 18 °C; at this temperature the r and GP values decreased by 18% and 54%, respectively. The decrease of these parameters can be explained as a disordering effect of proparacaine on the acyl chain and polar headgroup packing arrangements of the erythrocyte membrane. On the other hand, an increase of r implies that an ordering effect was taking place among the acyl chains, whereas higher values of GP can be interpreted as a decrease in the molecular dynamics or water penetration at the phospholipid headgroup region as a consequence of their interactions with proparacaine.

Table II. Effect of proparacaine on the anisotropy (r) of DPH and the general polarization (GP) of laurdan embedded in large unilamellar DMPC vesicles (LUV) at 37 °C and 18 °C.

Proparacaine (mM)	r (DPH)	GP (laurdan)	Temp. (°C)
0	0.079 ± 0.001	-0.074 ± 0.001	37
0.9	0.099 ± 0.001	0.002 ± 0.001	37
2.0	0.111 ± 0.004	0.043 ± 0.005	37
3.7	0.119 ± 0.007	0.057 ± 0.002	37
6.1	0.132 ± 0.010	0.083 ± 0.005	37
0	0.304 ± 0.001	0.474 ± 0.006	18
10.4	0.300 ± 0.002	0.389 ± 0.017	18
15.3	0.291 ± 0.002	0.340 ± 0.010	18
19.9	0.288 ± 0.002	0.309 ± 0.011	18
28.8	0.248 ± 0.002	0.220 ± 0.008	18

#### X-ray diffraction studies of phospholipid multilayers

Figure 4A shows the results obtained after DMPC was mixed and had interacted with water and proparacaine solutions. As expected, pure water altered the structure of DMPC: its bilayer width expanded from about 5.5 nm when dry to 6.45 nm when immersed in water and the reflections were reduced to only the first three orders of the bilayer width (Suwalsky, 1996). On the other hand, a new and strong reflection of 0.42 nm showed up. The appearance of this reflection was indicative of the fluid state reached by DMPC bilayers. In fact, it corresponds to the average separation of the fully extended acyl chains organized with rotational disorder in a hexagonal lattice. The

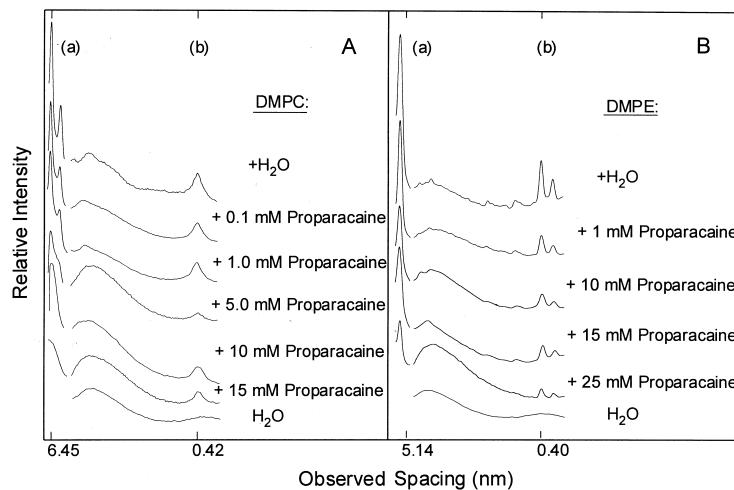


Fig. 4. Microdensitograms from X-ray diffraction diagrams of DMPC (A) and DMPE (B) in water and aqueous solutions of proparacaine; (a) low-angle and (b) high-angle reflections.

effects of proparacaine upon DMPC were concentration dependent: 0.1 mm caused a slight decrease of the low angle reflections (indicated as (a) in the figure) which correspond to the polarhead region; however, 5 mm proparacaine induced the disappearance of the low angle reflections which were replaced by a diffuse halo. On the other hand, no significant changes were observed in the high angle region (indicated as (b) in the figure) which corresponds to the acyl chain region of DMPC. These results imply that proparacaine induced serious molecular disorder of DMPC bilayers, especially in the polarhead groups. Figure 4B displays the results of the interaction of proparacaine with DMPE bilayers. As reported elsewhere (Suwalsky, 1996), water did not significantly affect the structure of DMPE. However, proparacaine increasing concentrations produced a progressive decrease in the reflection intensities of DMPE in both the low and high angle reflections; however, none of them vanished in any of the assayed concentrations of the anesthetic. To summarize, the LA interacted principally with the polar headgroups of DMPC perturbing their lattice arrangement, which was completely destroyed at a proparacaine concentration of 15 mm: at this concentration the LA induced milder structural perturbation of DMPE bilayers affecting their polar and acyl chain regions equally.

## Discussion

The main hypotheses that attempt to explain the molecular mechanisms of action of LA are: a) direct interaction with proteins, particularly  $\text{Na}^+$  voltage-gated channels (Li *et al.*, 1999), b) induction of structural alterations in their lipidic matrix, and c) action on the lipid-protein interfaces (Coutinho *et al.*, 1990). It is noteworthy that mechanism b) and c) involve nonspecific interactions of LA with membrane phospholipid bilayers. During the search for an *in vitro* system to examine the interaction of biologically relevant compounds with cell membranes, different cellular models have been applied. In our case we have been using the isolated toad skin, human erythrocytes and membrane molecular models. The toad skin is a useful model for studying the effect of LA on sodium absorption in tight epithelia. It is accepted that the decrease in the electrical parameters reflects inhibition

of active ion transport; the essential site of action of LA is the voltage-gated  $\text{Na}^+$  channel (Kuroda *et al.*, 1996; Li *et al.*, 1999; Nau *et al.*, 2000). Nevertheless, *Pleurodema thaul* skin possesses amiloride-sensitive  $\text{Na}^+$  channels (González *et al.*, 1989; Norris *et al.*, 1994) and the present results confirm the possibility that LA have important effects on ion channels of marked structural diversity. The finding that proparacaine was active at both surfaces of the skin suggests more than one site of action for the inhibitory effect of the anesthetic. This issue is partially resolved by X-ray diffraction, which showed that proparacaine perturbed the outer monolayer of the membrane phospholipids to a greater extent than those in the inner monolayer; in addition, fluorescence spectroscopy of IUM could imply proparacaine-protein interaction, and the formation of stomatocytes indicates that the anesthetic located in the inner moiety of the red cell membrane. Concentrations between 1–5 mm increased intracellular calcium ( $[\text{Ca}^{2+}]_i$ ) in corneal epithelial cells (Grant and Acosta, 1994); an increase in  $[\text{Ca}^{2+}]_i$  inhibits apical  $\text{Na}^+$  channels in frog skin epithelium (Brodin *et al.*, 1996). Enzyme activity is also affected: Igarashi *et al.* (1984) found that the degree of inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase activity is associated with the order of topical anesthetic activity in rabbit cornea. Skin membrane integrity was not affected by the proparacaine concentrations used since the current work showed that the resistance across the epithelium did not change significantly. The lasting nature of the skin response to proparacaine in spite of washout could be due to irreversible activation of protein kinase C, which in physiological conditions activates apical  $\text{Na}^+$  channels (Andersen *et al.*, 1990). Taken together, the results disclose proparacaine interaction with phospholipid bilayers, with ion channel proteins, and possibly more specifically, with both the outer and the cytoplasm mouth of the toad skin ENaC, all of which lead to decreased cell membrane functions.

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