Dibucaine-Induced Modification of Sodium Transport in Toad Skin and of Model Membrane Structures

Mario Suwalsky\textsuperscript{a,*}, Carlos Schneider\textsuperscript{a}, Fernando Villena\textsuperscript{b}, Beryl Norris\textsuperscript{b}, Hernán Cárdenas\textsuperscript{c}, Francisco Cuevas\textsuperscript{c}, and Carlos P. Sotomayor\textsuperscript{c}

\textsuperscript{a} Faculty of Chemical Sciences, University of Concepcion, Casilla 160-C, Concepcion, Chile. Fax: +56 41 245974. E-mail: msuwalsk@udec.cl
\textsuperscript{b} Faculty of Biological Sciences, University of Concepcion, Concepcion, Chile
\textsuperscript{c} Institute of Chemistry, Catholic University of Valparaiso, Valparaiso, Chile

* Author for correspondence and reprint requests

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The interaction of the local anesthetic dibucaine with the isolated toad skin and membrane models is described. The latter consisted of human erythrocytes, isolated unsealed human erythrocyte membranes (IUM), large unilamellar vesicles (LUV) of dimyristoylphosphatidylcholine (DMPC) and phospholipid multilayers built-up of DMPC and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively. Results indicate a significant decrease in the potential difference (PD) and in the short-circuit current (Isc) after the application of dibucaine in toad skin, which may be interpreted as reflecting inhibition of the active transport of ions. This finding might be explained on the basis of the results obtained from fluorescence spectroscopy and X-ray diffraction studies on membrane models. In fact, dibucaine induced structural perturbations in IUM, DMPC LUV and phospholipid multilayers. Scanning electron microscopy revealed that dibucaine induced erythrocyte stomatocytosis. According to the bilayer couple hypothesis an echinocytic type of shape change would have been expected given the preferential interaction of dibucaine with DMPC. Although it is still premature to define the molecular mechanism of action of dibucaine, the experimental results confirm the important role played by the phospholipid bilayers in the association of the anesthetic with cell membranes.

Introduction

Local anesthetics (LA) prevent the generation and the conduction of the nerve impulse, their primary site of action being the cell membrane. These compounds block conduction by decreasing or preventing the large transient increase in the permeability of excitable membranes to Na\textsuperscript{+} that is normally produced by threshold depolarization of the membrane (Catterall \textit{et al.}, 1996). The main hypotheses that attempt to explain their molecular mechanisms of action are: a) direct interaction with proteins, particularly Na\textsuperscript{+} voltage-gated channels (Li \textit{et al.}, 1999), b) induction of structural alterations in their lipidic matrix, and c) action on the lipid-protein interfaces (Coutinho \textit{et al.}, 1990). It is noteworthy that mechanisms b) and c) involve nonspecific interactions of LA with membrane phospholipids. On the other hand, it has been reported that structural perturbations induced to phospholipids in the neighborhood of ion channels affect channel activity (Martinac \textit{et al.}, 1990; Mouritsen and Jorgensen, 1992). Moreover, in order to reach the binding sites of sodium channels as stated by hypothesis (a) LA must diffuse across the membrane lipid bilayers.

Although the molecular mechanism of LA action is still not well understood, their lipophilicity make lipid-rich membranes sensitive target sites for their interaction with living organisms. In fact, it has been reported that they bind to a variety of cell membranes and induce functional perturbation of membrane proteins such as calmodulin, Na\textsuperscript{+} and K\textsuperscript{+} channels, acetylcholine receptors, AT-
Pases, 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 interactions (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 (Shimooka et al., 1992; Ueda et al., 1994; Kaneshina et al., 1997). For these reasons we thought it of interest to study the binding affinities of LA with cell membranes, their perturbing effects upon the phospholipid bilayer structures and the possible consequent alteration of ionic channel functions.

This article describes the interaction of the local anesthetic dibucaine with cell membranes. It is currently available as a spinal anesthetic and for topical use on the skin; nevertheless it is toxic, inducing neuroblastoma cell death due to membrane damage (Kim et al., 1997) and apoptosis in leukemic cells (Arita et al., 2000) by activation of caspases, a family of proteases which disassemble a cell by cleaving a set of proteins (Faleiro and La-zebnik, 2000). These effects indicate that dibucaine induces a variety of alterations in membrane transport systems. Like most LA, dibucaine is a tertiary amine, which at neutral pH exists in equilibrium between a neutral and a charged form. 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. Research in amphibian skin (Rytved et al., 1995; Ussing et al., 1996) has shown that the electrical properties of the toad skin are due principally to active Na\(^+\) transport from the outer (mucosal) to the inner (serosal) surface; this ion is exchanged for K\(^+\) by means of Na\(^+\) (K\(^-\))-ATPase. The short-circuit current (Isc), which is the amount of current necessary to bring the potential difference (PD) across the skin down to zero, measures the net transepithelial Na\(^+\) transport across the epithelium (Nielsen, 1997). The interaction of dibucaine with human erythrocytes was examined by phase contrast and scanning electron microscopy (SEM). These systems have been extensively used to determine the membrane-perturbing effects of numerous agents (Suwalsky et al., 1998; Suwalsky et al., 1999; Suwalsky et al., 2000). The cell membrane molecular models consisted in isolated human erythrocyte membranes (IUM), large unilamellar vesicles (LUV) of dimyristoylphosphatidylcholine (DMPC) and multilayers of DMPC and dimyristoylphosphatidylethanolamine (DMPE). They represent phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively. These systems have 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), pesticides (Suwalsky et al., 1998; Suwalsky et al., 1999) and metallic ions (Suwalsky et al., 1999).

Materials and Methods

Electrophysiological measurements on the isolated toad skin

The experiments were performed on samples of the abdominal skin dissected from anesthetized and pithed Pleurodema thaul toads of either sex (10–22 g). The amphibians were collected in fresh water ponds, fed on sow bugs (Oniscus asellus) and kept in tap water 24 h prior to use. Skins were mounted between two halves of a Ussing perspex chamber (Ussing, 1994); a circular area of 1.0 cm\(^2\) was exposed to 3.0 ml Ringer’s solution on each side. The composition of the solution was (mm): Na\(^+\) 114, K\(^+\) 2.5, Cl\(^-\) 117.5, Ca\(^{2+}\) 2.0, HCO\(_3^-\) 2.3, glucose 11, and oxygenated with a model Elite Hagen aerator. The Isc 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 monitored on a 2-channel recorder (Cole-Parmer, Chicago, IL, USA). 30 min after steady readings had been obtained, dibucaine was applied in the solution bathing
either the outer or the inner surface of the skin in the final concentrations specified in the text. The experiments were carried out at room temperature (18–22 °C). Results are expressed as means ± S. E. M. Student’s paired t test was used for statistical analysis.

Scanning electron microscopy (SEM) studies on human erythrocytes

*In vitro* interaction of dibucaine 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 lobule disinfected with 70% ethanol by aspiration into plastic tuberculin syringes without needles, containing 50 U/ml heparin in saline solution (0.9% NaCl). Red blood cells were then centrifuged, washed twice in saline, resuspended in buffer (7.5 mM phosphate, 145 mM NaCl, 5 mM glucose, 1 mM MgSO₄, pH 7.4) containing dibucaine at a final 1.5 mM concentration and incubated for 1 h at 37 °C. Controls were erythrocytes resuspended in incubation buffer without dibucaine. 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 10⁻¹ Torr 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 dibucaine 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 fluorescence steady-state 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 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., Cambridge, 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 (I.S.S. Inc., Champaign, IL, USA) respectively, both interfaced to computers. Software from I.S.S. 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., 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. Laurdan fluorescence spectral shifts were quantitatively evaluated using the GP concept (see above) which is defined by the expression 

\[ GP = \frac{(I_b - I_r)}{(I_b + I_r)} \]

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 (Parasassi et al., 1990). Dibucaine was incorporated in IUM and LUV suspensions by addition of small aliquots of a concentrated 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. Data given in Tables I and II represent mean values and standard error of ten measurements in two independent samples.

**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 dibucaine hydrochloride (lot 28H0354, MW 379.9) 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 \( \mu l \) of aqueous solutions of dibucaine in a range of concentration from 1 mM to 10 mM. They were X-ray diffracted two days after preparation 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 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 dibucaine applied in either the outer or the inner bathing solutions consisted of a concentration-dependent decrease in PD and Isc. Figure 1A shows that a 0.72 mM concentration was followed by a rapid decline of the electric parameters which reached a trough in 59.5 ± 3.4 min (n = 22) and was only partially reversible after washout of the skin (Fig. 1B). For both surfaces of the skin, a 0.48 mM concentration of the anesthetic reduced the electric parameters by over 35% (Figs 2A and B). The maximal concentration (0.72 mM) applied to the outer surface elicited a 42% decrease in Isc, and when applied to the inner surface was followed by a 74% decrease in Isc. In both cases, a moderate (17.0 ± 3.4%) although not significant (P >0.05, n = 19) increase in resistance was observed.

**Scanning electron microscope (SEM) studies of human erythrocytes**

Human red blood cells were incubated with 1.5 mM dibucaine. The phase contrast and SEM observations indicated that dibucaine induced a significant change in the shape of the erythrocytes. Apparently, the erythrocytes underwent a morphological alteration as they changed their discoid shape to spherostomatocytes. This result agreed with that published elsewhere (Malheiros et al., 2000). Some spherocytic forms and cell membrane disruption were also observed. 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 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 dibucaine produced stomatocytes would indicate that the anesthetic was located in the inner moiety of the red cell membrane.
Fig. 1. Single experiment illustrating the time course of the effect of dibucaine applied in the outer (A) and in the inner (B) bathing solution on the electric properties of the isolated toad skin. Isc = short-circuit current; PD = potential difference; W = washout. Inset: structural formula of dibucaine.

Fig. 2. Effects of increasing concentrations of dibucaine on the electric properties of the isolated toad skin. Results are expressed as percentage decrease in control values. Each point represents means ± S. E. M.; PD = potential difference; Isc = short-circuit current. Values for untreated skins were: PD 43.7 ± 3.5 mV and Isc 61.0 ± 4.1 μA/cm². Figures in parentheses refer to the number of experiments for each mean. A) outer surface, dibucaine 0.12, 0.24, 0.48 and 0.72 mM; B) inner surface, dibucaine 0.06, 0.12, 0.26, 0.48 and 0.72 mM. Significance by Student's paired t test: *P < 0.05; **P < 0.01; ***P < 0.03; ****P < 0.001; NS = not significant.
Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)

The structural effects of dibucaine on DMPC LUV were determined at the acyl chain hydrophobic core and at the hydrophilic/hydrophobic interface regions of the phospholipid bilayer by evaluation of DPH steady state fluorescence anisotropy ($r$) and laurdan general polarization (GP), respectively. Table I shows that increasing concentrations of dibucaine (0 mM to 0.141 mM) decreased $r$ values of DMPC LUV by an order of 16% at 18 °C, whereas there were no significant changes at 37 °C. Table II shows that the incorporation of dibucaine into the erythrocyte membrane (IUM) at 37 °C produced a 22% decrease in $r$ values. The decrease of this parameter, which was more significant for the erythrocyte membrane, can be explained as a disordered effect of the anesthetic on the acyl chain packing. On the other hand, the increase of laurdan GP indicated a decrease of the molecular dynamics or water penetration at the phospholipid polar group level. Results were more significant in LUV at 37 °C with increased GP values of the order of 50% (Table I) and 22% in IUM (Table II), whereas there was no significant increase of the GP values in LUV at 18 °C (Table I). These results suggest that dibucaine produced disordering effects at the phospholipid acyl chain level and reduced the hydration surrounding the head groups of membrane phospholipids. The water molecules participate in a hydrogen bond network between the phospholipid polar head groups and play an important role in bilayer stability. Studies provide evidence that acyl chains and head groups are structurally uncoupled; consequently, there is no correlation between head group hydration and lipid packing order (Ho et al., 1995).

X-ray diffraction studies of phospholipid bilayers

The molecular interactions of dibucaine with multilayers of the phospholipids DMPC and DMPE in an aqueous medium were determined by X-ray diffraction. Fig. 3A shows a comparison of the diffraction patterns of DMPC alone and of DMPC incubated with 1, 3 and 6 mM dibucaine. As expected, water altered the structure of DMPC: its bilayer width increased from about 5.5 nm in its dry crystalline form (Suwalsky, 1996) to 6.4 nm when immersed in water, and its reflections were reduced to only the first three orders of the bilayer width. On the other hand, a new and strong reflection of 0.42 nm 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 dibucaine produced a slight decrease in the phospholipid reflection intensities. However, 3 mM dibucaine induced a marked decrease of the 0.42 nm reflection intensity and the complete disappearance of the low angle reflections (indicated as (a) in the figure) which were replaced by a diffuse halo. This pattern remained practically unchanged with 6 mM dibucaine. These results imply that dibucaine induced serious molecular disorder of the DMPC bilayer, especially in the region of the polar head groups.

Fig. 3B shows the results of the interaction of dibucaine with DMPE. The perturbing effect of
this compound upon the structure of DMPE bilayers was milder than that observed in DMPC. In fact, a significant change in the lipid pattern was observed only at a 6 mM dibucaine concentration. This change consisted of a 50% reduction of the reflection intensities, which affected almost equally the low and high angle reflections (indicated as (a) and (b) in the figure, respectively). 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 in their dry crystalline phases (Suwalsky et al., 1996) 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 5.45 nm when dry up to about 6.3 nm when it is fully hydrated. This phenomenon allows the incorporation of dibucaine into DMPC bilayers producing its structural perturbation and complete destruction at a 3 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 (Suwalsky, 1996) nor by a number of compounds (Suwalsky et al., 1994). However, this organization did not prevent 6 mM dibucaine from interacting with and perturbing its structure.

**Discussion**

Results of the present study indicate that the significant decrease in the PD and in the Isc after the application of dibucaine to the toad skin may be interpreted as reflecting inhibition of the active transport of ions, since it has been shown that the Isc across isolated toad skin is equal to the net transepithelial transport (Nielsen, 1997). In accordance with mechanism (a) set up in the Introduction, there is evidence that the essential site of action of local anesthetics is the Na$^+$ channel (Kuroda et al., 1996; Nau et al., 2000). The observation that dibucaine was active at both surfaces of the skin suggests membrane permeation by the anesthetic, which shows a strong tendency to partition into lipid (de Paula and Schreier, 1996) changing the conformation of the lipid molecules and triggering structural changes in proteins (Seelig et al., 1988). Mechanism (b) is important since X-ray diffraction and fluorescence studies in the current work showed marked structural perturbation and possible destabilization of the DMPC bilayer structure. Changes in the lipid matrix will deform the lipid-protein interface (mechanism c); therefore dibucaine-induced changes of the phospholipid residing around the Na$^+$ channel protein (Wakita et al., 1992) make the anesthetic an effector of allosteric gating of the channel (Balser et al., 1996) because it interacts with hydrophobic amino acids such as phenylalanine present in the linker between two domains of the channel protein, leading to inactivation of the channel (Kur-
oda et al., 1996). It was surprising to find that the maximal concentration of dibucaine (0.72 mM) was more effective when applied to the inner surface of the skin, especially as in our experiments the DMPE bilayer was affected only at anesthetic concentrations far higher (6 mM) than those used on the skin. Three explanations might be suggested: 1) dibucaine inhibits dog kidney Na⁺,K⁺-ATPase activity (Hudgins and Bond, 1984); 2) as discussed later in this work, if the location of dibucaine in the inner moiety of the erythrocyte membrane activates lipid scrambling, this could contribute to collapse lipid asymmetry required for normal membrane function; 3) the hydrophobic binding domain might be responsible for channel inactivation from the cytoplasmic mouth of the Na⁺ channel (Zamponi and French, 1994). The persistent decline of the electric properties of the skin in spite of washout could be due to anesthetic-induced changes of the characteristics of the lipid boundary domains of the integral proteins. The maximal concentration used on the skin did not cause disruption of membrane integrity, as this would involve decreased resistance across the bilayer, which was not the case.

Electron microscopy observations of human erythrocytes incubated with dibucaine indicated that the anesthetic was probably located in the inner moiety of the red cell membrane. However, X-ray diffraction of phospholipid bilayers showed that dibucaine most likely interacted with the lipids located in the outer monolayer of the erythrocyte membrane. The explanation for this discrepancy with the bilayer couple hypothesis could be based on the “lipid scrambling mechanism” proposed by Schrier et al. (1992). According to them, some cationic amphipaths produce a rapid scrambling of the erythrocyte bilayer with phosphatidylycholines (PC) and sphingomyelins (SM) moving inward while phosphatidylethanolamines (PE) move outward along with phosphatidylycerines (PS). Thus, the interaction of dibucaine 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., 1992). It is noteworthy that the results obtained by fluorescence spectroscopy in DMPC LUV tend to agree with those revealed by X-ray diffraction in DMPC multilayers. In conclusion, our results unambiguously showed that dibucaine interacted with phospholipid bilayers, particularly with PC. Therefore, cell membrane structure and physiological properties such as fluidity, permeability, receptor and channel functions may be affected.

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