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Effects of the local anesthetic benzocaine on the human erythrocyte membrane and molecular models

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Abstract

The interaction of the local anesthetic benzocaine with the human erythrocyte membrane and molecular models is described. The latter consisted of isolated unsealed human erythrocyte membranes (IUM), large unilamellar vesicles (LUV) of dimyristoylphospatidylcholine (DMPC), and phospholipid multilayers of DMPC and dimyristoylphospatidyletanolamine (DMPE), representatives of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively. Optical and scanning electron microscopy of human erythrocytes revealed that benzocaine induced the formation of echinocytes. Experiments performed on IUM and DMPC LUV by fluorescence spectroscopy showed that benzocaine interacted with the phospholipid bilayer polar groups and hydrophobic acyl chains. X-ray diffraction analysis of DMPC confirmed these results and showed that benzocaine had no effects on DMPE. The effect on sodium transport was also studied using the isolated toad skin. Electrophysiological measurements indicated a significant decrease in the potential difference (PD) and in the short-circuit current (Isc) after the application of benzocaine, reflecting inhibition of active ion transport.

Keywords: Local anesthetic; Benzocaine; Erythrocyte membrane; Phospholipid bilayer

1. Introduction

Benzocaine, a local anesthetic, is an ester of paraaminobenzoic acid (ethyl *p*-aminobenzoate, Fig. 1), poorly soluble in water and too slowly absorbed to be toxic. When applied to wounds and ulcerated surfaces, it remains localized for long periods of time to produce a sustained anesthetic action [1]. The molecular mechanism of action of the local anesthetics (LA) has not yet been quite established [2]; however, their lipophilicity make lipid-rich membranes sensitive targets for their interaction with living organisms. The main hypotheses that attempt to explain the action of LA are: (a) direct interaction with proteins, particularly Na^+ voltage-gated channels [3–5], (b) induction of structural alterations in membrane

Abbreviations: SEM, scanning electron microscopy; LUV, large unilamellar vesicles; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; PD, potential difference; Isc, short-circuit current.

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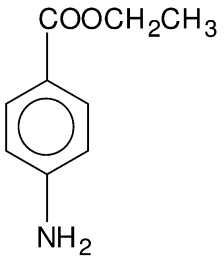

Fig. 1. Structural formula of benzocaine.

lipid matrix [6,7], and (c) action on lipid–protein interfaces [8]. Na^+ channels are responsible for the initiation and propagation of action potentials in most excitable cells [9,10]. Local anesthetics prevent the generation and the conduction of the nerve impulse by decreasing or preventing the large transient increase in the permeability of excitable membranes to Na^+ that is normally produced by threshold depolarization of the membrane [1], but also interact with other cellular systems [11]. Examples are the K^+ channels [12], Ca^{2+} channels [13], γ-aminobutyric acid receptors [14], Na, K-ATPase [15], Ca-ATPase [16], G Protein [17] and nicotinic acetylcholine receptors [18]. It has been suggested that changes in the molecular organization of membranes such as increased fluidity are involved in the mechanism of anesthesia. It has also been reported that structural perturbations of phospholipids in the neighborhood of ion channels affect channel activity [19,20]. 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 [21]. For these reasons we thought it of interest to study the binding affinity of the local anesthetic benzocaine with cell membranes, its perturbing effect upon phospholipid bilayer structures and the possible consequent alteration of ionic channel functions. With this aim we used (a) human erythrocytes, which were observed by phase contrast and scanning electron microscopy (SEM); (b) isolated unsealed human erythrocyte membranes (IUM), studied by fluorescence spectroscopy; (c) bilayers of dimyristoylphosphatidycholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), in representation of phospholipid classes located in the outer and inner monolayers of cell membranes, respectively [22], particularly of the human erythrocyte [23], respectively, and analyzed by X-ray diffraction; (d) large unilamellar vesicles (LUV) of DMPC, examined by fluorescence spectroscopy, and (e) isolated toad skin, a biological model of sodium–transporting membranes [24,25], which was subjected to electrophysiological measurements. All these systems have been used to determine the interaction with and perturbing effects on membranes by the LA dibucaine [26], propara-caine [27] and bupivacaine [28].

2. Materials and methods

2.1. Phase contrast and SEM studies on human erythrocytes

In vitro interaction of benzocaine 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 centrifuged, washed twice in saline, resuspended in buffer (7.5 mM phosphate, 145 mM NaCl, 5 mM glucose, 1 mM $MgSO_4$, pH 7.4) containing benzocaine at concentrations equivalent to 3 and 10 mM, and incubated 1 h at 37 °C. Sample drops were then examined in a phase contrast microscope (Leitz Orthoplan, Wetzlar, Germany). Controls were erythrocytes resuspended in incubation buffer without benzocaine. Red blood cells were immediately fixed overnight at 5 °C by adding one drop of each sample to plastic tubes containing 1 ml 2.5% glutaraldehide in saline. Erythrocytes were afterwards 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 Corporation, Hayward, CA, USA).

2.2. Fluorescence measurements of IUM and DMPC LUV

The influence of benzocaine 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 fluorescent 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 on the dynamic properties at the level of the lipid polar head organization in the zone of the erythrocyte membrane and in DMPC LUV. Laurdan fluorescence spectral shifts were quantitatively evaluated using the general polarization (GP) concept [29] which is defined by the expression $GP=(I_b-I_r)/(I_b+I_r)$, where I_b and I_r are the emission intensities at the blue and red edges of the emission spectrum, respectively. These intensities have been measured at the emission wavelengths of 440 and 490 nm, which correspond to the emission maxima of laurdan in the gel and liquid crystalline phases, respectively [30]. DPH fluorescence anisotropy (r) was calculated according to the definition: $r=(I_v-I_h)/(I_v+2I_h)$, where I_v and I_h are the corresponding vertical and horizontal polarized emission fluorescence intensities [31]. The emission was measured using a WG-420 Schott high-pass filter (Schott WG-420, Mainz, Germany) with negligible fluorescence.

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. [32]. 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 Corporation, 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 the addition of small aliquots of concentrated solutions of the probe in dimethylformamide and ethanol, respectively, and incubated at 37 °C for 1 h. Fluorescence spectra and anisotropy measurements were performed on a Spex Fluorog (Spex Industries Inc., Edison, NJ, USA) and in a phase shift and modulation Gregg-200 steady-state and time resolved spectrofluoremeter (ISS Inc., Champaign, IL, USA), respectively, both interfaced to computers. Software from ISS was used for data collection and analysis. Measurements of IUM were made at 37 °C while those of DMPC LUV suspensions were made at 18 and 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). Benzocaine was incorporated in IUM and LUV suspensions by addition of appropriate aliquots in ethanol in order to obtain the different concentrations used in this work. Control experiments to assess the influence of dimethylformamide and ethanol on the anisotropy and GP of samples were performed; the results indicated that at the actual concentrations of 12.9 mM and 17.5 mM, respectively, no effects were apparent. Significant effects were observed only at concentrations above 50 mM. Blank subtraction was performed in all measurements using labeled samples without probes. The data presented in Tables 1 and 2 represent mean values and standard errors of 10 measurements in two independent samples. Unpaired Student's t-test was used for statistical calculations.

2.3. 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 benzocaine (lot 128H1235, MW 165.2)

Table 1
Effect of benzocaine on the anisotropy (r) of DPH and the general polarization (GP) of laurdan embedded in isolated unsealed human erythrocyte membrane (IUM) at 37 °C

Benzocaine (mM)	r (DPH)	GP (laurdan)
0.0	0.291 ± 0.001	0.283 ± 0.003
0.15	0.283 ± 0.002	0.278 ± 0.003
1.1	0.212 ± 0.003	0.264 ± 0.001
3.2	0.204 ± 0.001	0.256 ± 0.004
5.6	0.198 ± 0.002	0.245 ± 0.005
10.0	0.170 ± 0.002	0.210 ± 0.002
20.0	0.145 ± 0.005	0.178 ± 0.002

from Sigma (MO, USA) were used without further purification. About 3.5 mg of each phospholipid was mixed with different amounts of benzocaine in a mortar and translated into 2.0-mm-diameter glass capillaries (Glas-Technik and Konstruktion, Berlin, Germany) to which 200 μl of bidistilled water were added to obtain a concentration of benzocaine in a range equivalent to 10–50 mM. The specimens 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 CuKα radiation from a Philips PW 1140 X-ray generator (Eindhoven, Netherlands) was used. The relative reflection intensities on films 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 temperature of both DMPC and DMPE. Higher temperatures would have induced transitions to more fluid phases, making the detection of structure changes harder.

2.4. Electrophysiological measurements on the isolated toad skin

Skins from toads of either sex (Pleurodema thaul, 10–20 g) collected from fresh water ponds were used. The toads were kept at room temperature in bins containing tap water 24 h prior to use and fed on sow bugs (*Oniscus asellus*). The toads were pithed and samples of the abdominal skin were removed, washed in toad Ringer's solution and mounted between two halves of a perspex Ussing-type chamber. A circular area of 1.00 cm^2 was exposed to 3.0 ml Ringer' solution on each side. The composition of the solution was (in mM): Na^+ 114, K^+ 2.5, Cl^- 117.5, Ca^{2+} 2.0, HCO_3^- 2.3 and glucose 11, and was oxygenated by means of an Elite 800 aerator (R.C. Hagen, Mansfield, MA). All experiments were carried out at room temperature (18–22 °C). The Isc was

Table 2
Effect of benzocaine on the anisotropy (r) of DPH and the general polarization (GP) of laurdan embedded in large unilamellar DMPC vesicles at 18 and 37 °C

Benzocaine (mM)	r (DPH)	GP (laurdan)	Temperature (°C)
0.0	0.304 ± 0.001	0.500 ± 0.001	18
0.15	0.285 ± 0.006	0.494 ± 0.002	18
1.1	0.216 ± 0.001	0.390 ± 0.003	18
3.2	0.192 ± 0.007	0.312 ± 0.004	18
5.6	0.174 ± 0.002	0.232 ± 0.004	18
10.0	0.168 ± 0.003	0.179 ± 0.003	18
20.0	0.111 ± 0.007	–	18
0.0	0.077 ± 0.002	−0.064 ± 0.004	37
0.15	0.078 ± 0.002	−0.074 ± 0.004	37
1.1	0.095 ± 0.005	−0.077 ± 0.001	37
3.2	0.105 ± 0.002	−0.074 ± 0.001	37
5.6	0.097 ± 0.002	−0.068 ± 0.002	37
10.0	0.099 ± 0.003	−0.056 ± 0.004	37
20.0	0.091 ± 0.006	−0.037 ± 0.011	37

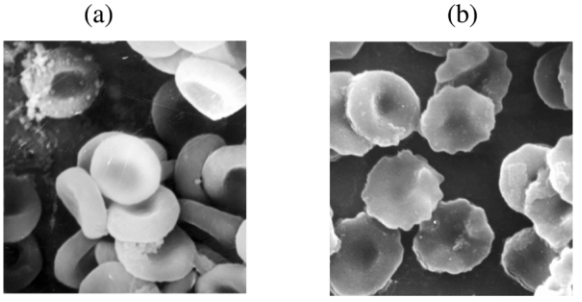

Fig. 2. Effect of benzocaine on morphology of human erythrocytes. Images (obtained by scanning electron microscopy) of (a) untreated erythrocytes (2900×) and (b) erythrocytes incubated with 10 mM benzocaine (2700×).

monitored with non-polarizable Ag/AgCl electrodes placed at 15-mm distance from the skin and connected to a CH 1023 voltage-clamp circuit (G. Métraux Electronique, Crissier, Switzerland) set to keep the PD across the skin at 0 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 (Chicago, IL) recorder. Thirty minutes after steady readings had been obtained, benzocaine 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 ± S.E. Student's paired *t*-test was used for statistical analysis.

3. Results

3.1. Phase contrast and SEM studies on human erythrocytes

Human red blood cells were incubated with concentrations equivalent to 3 and 10 mM benzocaine. Phase contrast and SEM observations indicated that only 10 mM benzocaine induced a significant change in the shape of the erythrocytes. In fact, the erythrocytes underwent a morphological alteration as they changed their discoid shape (Fig. 2a) to echinocytes (Fig. 2b). According to the bilayer couple hypothesis [33] 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 benzocaine induced the formation of echinocytes indicate that the anesthetic was located in the outer moiety of the red cell membrane.

3.2. Fluorescence measurements of IUM and DMPC LUV

The structural effects of benzocaine on IUM were determined at 37 °C at the acyl chain hydrophobic core and at the hydrophilic/hydrophobic interface regions of the membrane bilayer by evaluation of DPH steady-state fluorescence anisotropy (r) and laurdan GP, respectively. Table 1 shows that the incorporation of benzocaine in the range of 0–20 mM produced a 50% reduction of r and a 37% decrease of the GP. The decrease of r and GP can be explained as a disordering effect of the anesthetic on the order of both acyl chain and polar groups of the erythrocyte membrane. Table 2 shows that increasing concentrations of benzocaine (0–20 mM) at 18 °C decreased r and GP values by approximately 64%, which can be interpreted as a decrease in the order on the acyl chain and polar groups packing arrangement of DMPC. However, at 37 °C there was an increase of 18% and 42% in r and GP values, respectively, in the 0–20 mM concentration range. 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 interaction with benzocaine.

3.3. X-ray diffraction studies of phospholipid multilayers

The molecular interactions of benzocaine 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 benzocaine in concentrations equivalent to 10 and 50 mM. As expected,

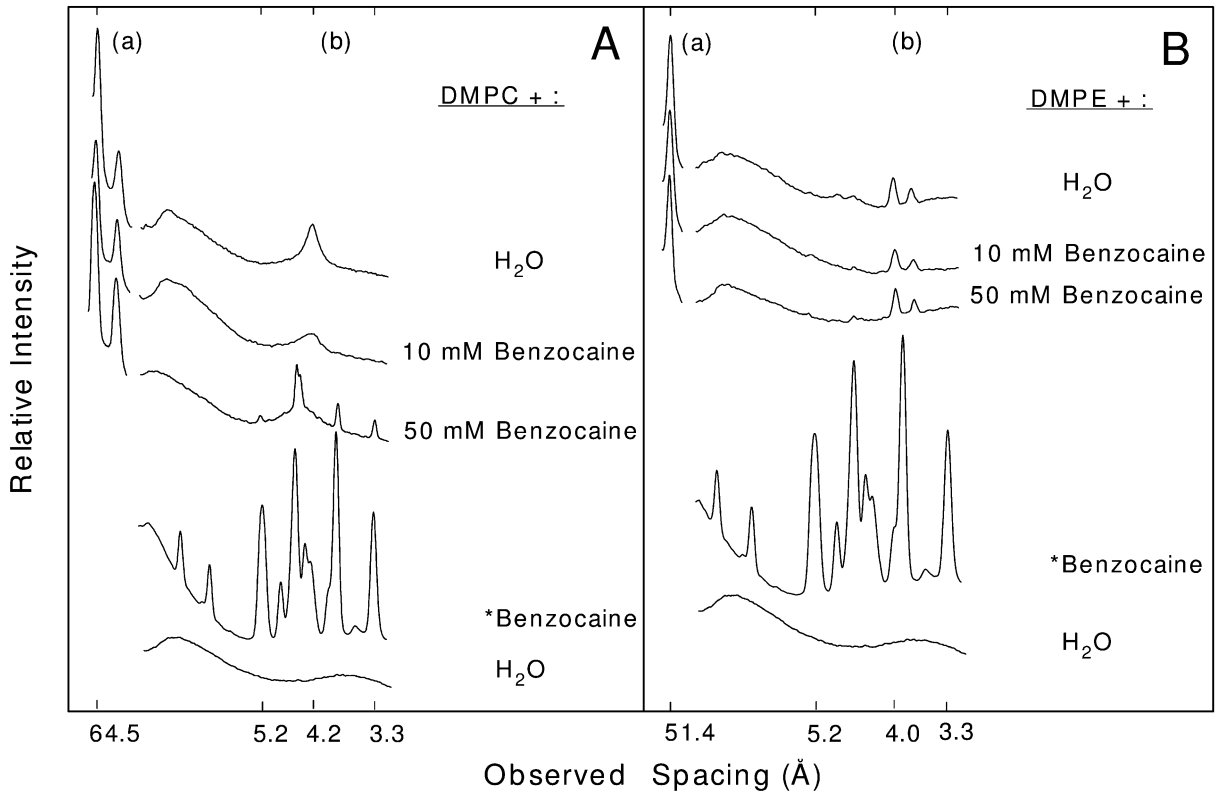

Fig. 3. Microdensitograms from X-ray diffraction diagrams of DMPC (A) and DMPE (B) in water and in aqueous suspensions of benzocaine; (a) low-angle and (b) high-angle reflections. *Dry specimen.

water altered the structure of DMPC: its bilayer width increased from 55 Å in its dry crystalline form [34] to 64.5 Å when immersed in water, and its reflections were reduced to only the first 3 orders of the bilayer width. However, a new and strong reflection of 4.2 Å showed up, whose appearance was indicative of the fluid state reached by DMPC bilayers and corresponded to the average distance between its fully extended acyl chain organized with rotational disorder in hexagonal packing. Addition of 10 mM benzocaine caused a marked decrease in the high angle 4.2 Å reflection intensity, which practically disappeared when the anesthetic concentration reached 50 mM. The low angle phospholipid reflection intensities (indicated as (a) in the figure), remained mostly unchanged after exposure to benzocaine (10–50 mM). These results imply that benzocaine induced serious molecular disorder in the region of the acyl chain of DMPC. Fig. 3b shows the outcome of the interaction of benzocaine with DMPE. As reported elsewhere [34], water did not significantly affect the structure of DMPE. The perturbing effect of benzocaine upon the structure of DMPE was practically negligible even at a 50 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 in their dry crystalline phases [34] with the hydrocarbon chains mostly parallel and extended, and the polar groups lying perpendicular to them. However, the gradual hydration of DMPC results in water filling the highly polar interbilayer spaces. This effect allows the incorporation of benzocaine into DMPC bilayers and subsequent structural

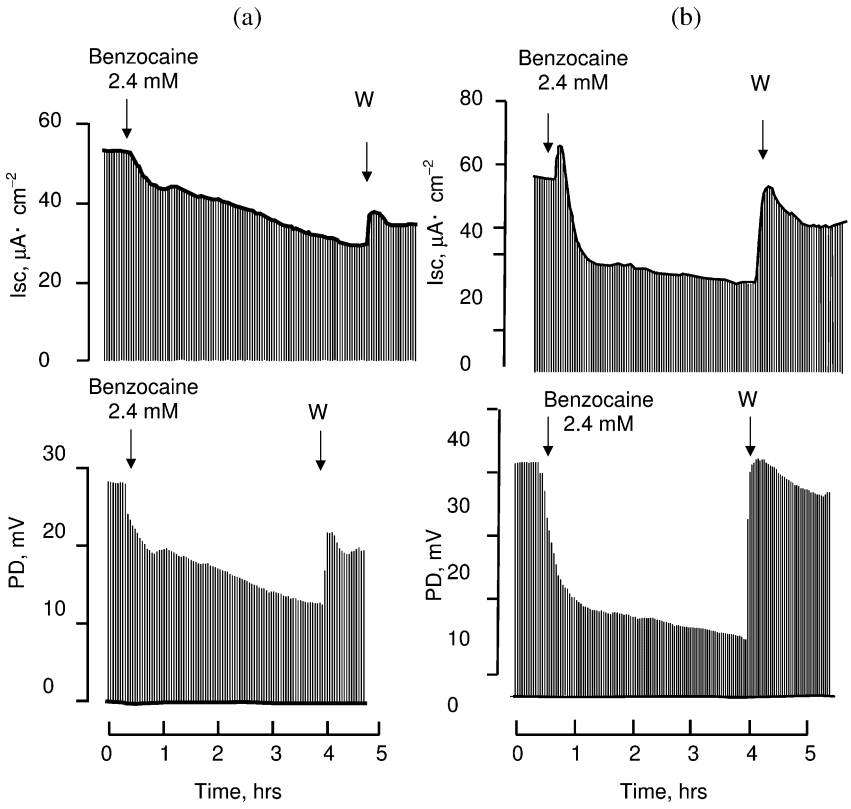

Fig. 4. Single experiment illustrating the time course of the effect of 2.4 mM benzocaine applied in the outer (a) and in the inner (b) bathing solution, on the electric parameters of the isolated toad skin. Isc = short-circuit current; PD = potential difference; W = washout.

perturbation of the phospholipid. 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 [34] nor by a number of compounds [35] including benzocaine.

3.4. Electrophysiological measurements on the isolated toad skin

Benzocaine (2.4 mM) applied in the solution bathing either the outside or the inside surface of the toad skin, after a latency period of 0.88 ± 0.03 min, $n = 18$, decreased Isc and PD; the electrical parameters reached a trough in approximately 26 min (Fig. 4a and b). These results probably reflect inhibition of transepithelial ion transport [24]. The effect was concentration-dependent (Fig. 5a and b) with a concentration for half-maximal response (EC_{50}) of approximately 0.24 mM, a value that may be compared with the 0.25 mM benzocaine reduction of INa to 40% of its basal value in nerve fibers of frog [36]. For the two larger concentrations (0.24 and 2.4 mM), the effect was greater when the anesthetic was applied to the inside surface. Complete reversibility of the effects was achieved by washout in 60% of the experiments indicating that in these trials membrane integrity was not disrupted. However, skin resistance decreased significantly at the maximal concentration used (15.0 ± 0.05, $P < 0.05$, $n = 18$) and this could indicate serious perturbation of membrane structure in 40% of the experiments.

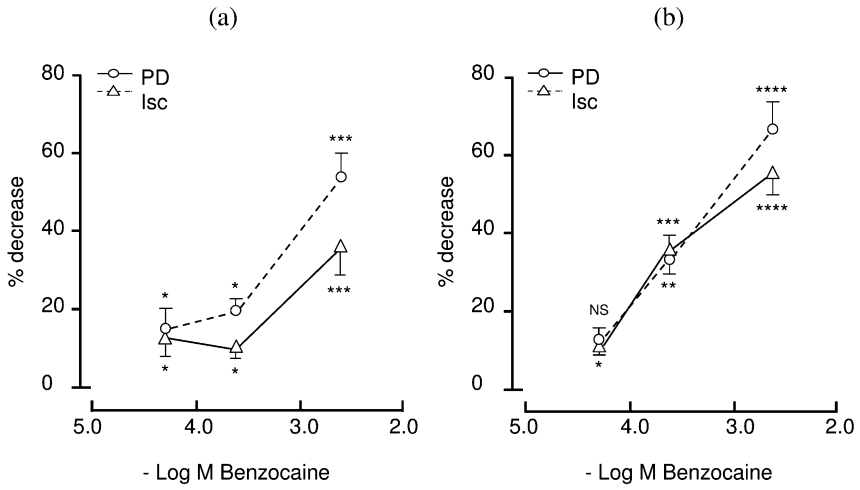

Fig. 5. Effect of benzocaine in increasing concentrations on the electric properties of the isolated toad skin. Potential difference (PD) and short-circuit current (Isc) values for untreated skins were 28.2±3.4 mV and 52.9±4.5 μA/cm², respectively. Results obtained in the presence of benzocaine (−log 0.048, 0.24 and 2.4 mM) are expressed as the percentage decrease of these control values. Each point represents means±S.E. Figures in parentheses refer to the number of experiments for each mean. (a) Benzocaine applied to the outside surface; (b) benzocaine 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.

4. Discussion

The induction of structural alterations in membrane lipid matrix (mechanism proposed in hypothesis b; see Section 1) is important since it involves nonspecific interactions of benzocaine with phospholipid bilayers. These interactions imply a decreasing membrane organization [37] that could affect the transport of Na^+ and K^+ in nerve membranes, leading to anesthetic action [7]. Optical and electron microscopy observations of human erythrocytes incubated with benzocaine confirmed that the anesthetic was located in the outer monolayer of the red cell membrane. This result agrees with those obtained by X-ray experiments, performed on bilayers made up of classes of the major phospholipids present in either the outer or inner sides of the erythrocyte membrane. In fact, they showed that 10 mM benzocaine disordered the acyl chain region of DMPC (the major class of lipid present in the outer monolayer of the erythrocyte membrane) and 50 mM almost completely perturbed it. A similar conclusion was derived from a ^2H NMR spectroscopic study [38]. Fluorescence spectroscopy of human erythrocyte membranes at 37 °C and DMPC LUV in the gel phase at 18 °C confirmed these results. In fact, the assays showed that benzocaine (0–20 mM) induced a significant reduction of their *r* and PG values, indicative of enhanced disorder at the acyl chain and polar head region of their lipid bilayers. However, at 37 °C (liquid crystalline phase) *r* and GP values of DMPC LUV increased after the addition of benzocaine (0–20 mM). The latter result implies that the anesthetic increased the order of the fluid phase present at this higher temperature. On the other hand, no significant effect was observed by X-ray diffraction of DMPE (which preferentially locates in the erythrocyte inner monolayer) at the highest assayed benzocaine concentration.

As described in previous papers [26–28], the decrease in the electrical parameters of the isolated toad skin induced by application of LA probably reflects inhibition of Na^+ transport across the epithelium. Although voltage-gated Na^+ channels are primary targets for local anesthetics [3], the anesthetic must first reach the channels. In addition, the effect of benzocaine on toad skin ion transport is due not to an action on a voltage-

sensitive channel but to an effect on targets such as: (a) proteins, particularly epithelial channels (ENaCs) which are mainly amiloride sensitive [39–41]; (b) membrane lipid monolayers [42]; and (c) specific membrane receptors [3,11]. Mechanism (a) is essential; these channels, expressed in many tissues, serve diverse functions including Na^+ absorption [43] and are present in Pleurodema thaul skin [39,44]. It is of importance to consider that the degenerin/epithelial sodium channel (Deg/ENaC) superfamily [41] has a physiological role in transepithelial salt and water movement, data that confirm the possibility that LAs are effective in ion channels of marked structural and functional diversity. Apparently, residues within the M2 domains of ENaC contribute to the channel's conduction pore and certain sites within the domain might confer ion selectivity [45]. Although inhibition of sodium channels in the cytoplasm is mainly induced by protonated charged anesthetics in physiological pH [46], uncharged species, including benzocaine, are of limited but measurable potency [47,48]. It is doubtful that this anesthetic acts on enzyme activity; benzocaine inhibited canine renal medulla ATPase activity only at 37 °C [15] and all our experiments on the amphibian skin were carried out at room temperature (see Methods). Mechanisms (b) and (c), although not specific, contribute at least partially to the effect of benzocaine. Evidence for mechanism (b) is the finding that benzocaine expanded lipid monolayers which mimick cell membrane [42], thus leading to changes in lipid–protein interfaces, which in turn affect (c) membrane receptors. Such a mechanism was found for muscle Na^+ channel in human embryonic kidney cells [3] where benzocaine blocked the putative LA receptor in D4-S6, and also for the inhibition of lysophosphatidic acid induction of Cl^- currents in *Xenopus oocytes* [11]; the membrane receptor was identified as the LA site of action. Experimental results showed that benzocaine locates in the external moiety of the erythrocyte membrane, whereas the inhibition of sodium transport in toad skin is more evident when the anesthetic is applied in its inside surface. A possible explanation for these apparently contradictory results may lie in the fact that the outer monolayer of the human erythrocyte is richer in lecithins than the inner one [23]. However, as the lipid distribution in the toad skin membrane is unknown, a higher concentration of lecithins in its inner monolayer, as reported for several other cell membranes [22] cannot be disregarded. In addition, the cornified epithelium covering the skin is an effective barrier whereas the inside or serosal barrier is weaker and, therefore chemical agents have easier access to the inner aspect of the skin [49].

We have found no explanation for the initial stimulatory effect of 0.24 mM benzocaine. It might be speculated that this particular concentration increases the permeability of possible amiloride-insensitive Na^+ channels in the toad skin; or, alternately, as shown for the tertiary amine procaine by Flonta et al. [40], that benzocaine in the outer bathing solution could transiently increase Cl^- conductance. To conclude: benzocaine decrease of the electrical parameters of the isolated toad skin may be interpreted as inhibition of Na^+ transport across the epithelium, due to expansion of the lipid bilayers, alteration in lipid–protein interfaces, membrane permeation, binding to membrane lipid and Na^+ channel receptors, and blockade of ENaC from both outer and inner surfaces of the epithelium.

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