

Effects of an Amphipathic Drug on the Rheological Properties of the Cell Membrane

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ABSTRACT: Sodium thiopental, as other amphiphilic molecules, interacts with the membrane by inserting into the lipid bilayer and causing alterations of the membrane properties such as curvature and hypotonic lysis resistance. But can it modify the mechanical properties of the membrane? In the present work it was observed that sodium thiopental affected the membrane rheological properties by improving erythrocyte deformability; this effect resulted from a reduction of both the elastic modulus and surface viscosity. In erythrocytes devoid of sialic acid after treatment with neuraminidase, sodium thiopental membrane concentration was significantly higher than in normal cells, suggesting that drug access to the lipid bilayer be facilitated by the absence of the steric and electrostatic barrier of the glycocalyx negative charges. From a rheological point of view, desialated and normal cells showed the same response to the anesthetic as regards elastic modulus but in opposite direction if surface viscosity was considered. This finding supports the hypothesis that sodium thiopental molecules enter the bilayer of desialated cells in a higher proportion, as compared to the normal erythrocyte, promoting a disorganization that results in a greater inner friction. The changes in the rheological parameters, triggered by sodium thiopental, could be attributed to the bilayer contribution to the membrane mechanical properties, either directly or through interaction between the bilayer and the cytoskeleton.

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INTRODUCTION

It is currently accepted that the isolated lipid bilayer is a fluid lacking shear rigidity and with negligible viscosity. In that respect, the membrane elastic modulus (μ) and viscosity (η) have been solely attributed to the cytoskeleton proteins, and the influence of the bilayer on the membrane mechanical properties and deformability has been disregarded (1).

Studies on cell deformability as a function of temperature (2) showed an inflexion at the transition temperature of the lipid phase, suggesting that the bilayer may play a role in membrane rheology. Clinical studies have demonstrated a reduced deformability associated

to alterations in the erythrocyte membrane lipid composition. Moreover, cholesterol accumulation increases membrane surface but decreases its deformability (3).

Amphiphilic molecules that interact with the membrane by inserting in the lipid bilayer, like, the anesthetic sodium thiopental, modify cellular shape (4), and, at low doses, protect the erythrocyte from hypotonic lysis (5,6,7). The mechanism by which they modify membrane stability is not completely understood; several studies ascribe the antihemolytic effect to an increase in the critical volume needed to produce hemolysis due to the membrane expansion and

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fluidification brought about by insertion of these molecules in the lipid bilayer (8,9).

To explore the probable role of the bilayer in membrane rheology, we analyzed the effect of sodium thiopental on red blood cells elastic modulus and surface membrane viscosity. These rheological parameters were measured in intact erythrocytes under controlled experimental conditions that allowed the assumption that no significant changes in cytoplasmic viscosity or in area/volume ratio occur. The changes on elastic modulus and surface viscosity coefficient in the presence of sodium thiopental could then be attributed to modifications of the membrane mechanical properties, in agreement with the proposed role of the bilayer. To determine the interaction between the anesthetic and the surface membrane charge, desialated erythrocytes treated with sodium thiopental were also studied. Our results suggest that drug entrance is facilitated in desialated cells, thus enhancing the effect on the elastic modulus and inducing molecular membrane disorganization manifested through increased inner friction.

MATERIALS AND METHODS

Erythrocytes

Blood samples were obtained from healthy adult volunteers by venous puncture and placed into heparinized tubes. The samples were centrifuged and washed three times with phosphate buffered saline (PBS), pH 7.4, at 4 C. RBC at 10% hematocrit were treated with 5 mM sodium thiopental (Abbott Lab. S.A.) (unless another concentration is shown) for thirty minutes at 37 C.

Rheological Parameters

Two parameters, the elastic shear modulus and the coefficient of surface viscosity, characterize erythrocyte deformability. Shear modulus provides a measure of the equilibrium extension of the membrane, while membrane

surface viscosity is an index of internal friction that delays shape change. An erythrodeformeter, based on Mohandas' method (10), using laser diffraction (11,12), was employed to determine these parameters. Briefly, RBC (4 to 8×10^7 cells/ml) in a high viscosity PVP solution (viscosity 12cp; 290 mOsm; pH 7.4) were submitted to shear stress (1.72×10^2 dynes/cm²) to achieve maximum deformation. Sodium thiopental concentration was maintained constant throughout the measurement. The laser beam passing through the suspension produced a diffraction pattern of deformed erythrocytes (ellipsoid). The two principal diameters of the pattern were measured with a photometric device.

The variables estimated were I. Deformation index (δ): the relationship between the major (T) and minor (L) axes of the ellipse ($\delta = (T-L)/(T+L)$), II. Membrane elastic modulus (μ), a measure of the magnitude of the force needed to induce uniaxial deformation at a constant area, i.e. shear stress/deformation index ratio, III. Time constant or time lag (t_r): the time constant of the deformation-time exponential function when the shear stress is suddenly interrupted, IV. Surface viscosity (η): the product of t_r and μ .

Osmotic Fragility Measurements

RBC samples, with and without sodium thiopental, were diluted either with water or 0.01 M PBS (0 to 0.145 M NaCl), to obtain an osmotic concentration ranging from 0 to 290 mOsm/kg and pH 7.4, (13). The sodium thiopental concentration remained constant in treated cells. After 30 minutes, the samples were centrifuged at $515 \times g$ for 5 minutes. Hemolysis percentage was determined photometrically at 540 nm, considering 100% hemolysis at 0 mOsm/kg.

Erythrocyte Shape Assessment

An aliquot of 1% v/v RBC suspension in saline (0-20mM sodium thiopental-treated cells) was placed on a vinyl plastic slide, and the cell shape was observed with an inverted microscope.

According to Bessis' classification (14) nine shapes could be distinguished: biconcave disc (shape index 0); three types of stomatocytes (indexes -1, -2 and -3); spherostomatocyte (index -4); three types of echinocytes (indexes 1, 2 and 3) and spheroechinocyte (index 4). Two hundred cells per slide were counted and classified, and the average index value was calculated.

Erythrocyte Surface Calculation

Washed erythrocytes were subjected to gradual hemolysis. The diameter of spherical swollen cells was measured using an Olympus OSM-D₂ micrometer. An average diameter was used to calculate the spherical surface.

Neuraminidase Treated Cells

Two hundred microliters of washed RBC were added to a neuraminidase solution (*Clostridium perfringens*, SIGMA type V, 22.4 µg/ml), incubated at 37 C for an hour with intermittent gentle shaking, and centrifuged at 1200 × g for 5 minutes. The sialic acid released was determined by the resorcinol colorimetric reaction (15) in the supernatant. Treated RBC were washed and suspended in saline, with or without sodium thiopental, for 30 minutes at 37 C, and submitted to the same procedures as non treated ones, to determine the rheological parameters and to analyze shape changes and osmotic fragility.

Assessment of Sodium Thiopental in the Red Blood Cell Membrane

Sodium thiopental determinations were carried out by spectrophotometry (16). The sodium thiopental bound to RBC membrane was assessed comparing its concentration in the sodium thiopental solution before and after incubating either RBC or ghosts (10⁶ cells/mm³). The supernatant volume was adjusted taking into account the intercellular-trapped fluid in the sodium thiopental-RBC suspension, by the inulin

method. The same experiment was performed with desialated cells.

Statistical Analysis

Student's *t* test for paired data was used to determine the difference between treated and untreated cells. Values are presented as mean ± standard error (SEM).

Sodium thiopental concentration medium difference, before and after incubation of either RBC or ghosts (both normal and desialated), was analyzed by the non parametric Wilcoxon's test for paired data (17). Values are presented as median and range.

The Ethical Committee of the Facultad de Ciencias Médicas de la Universidad Nacional de Rosario approved this research, and all the participants signed an informed consent.

RESULTS

Assessment of Sodium Thiopental in the Red Blood Cell Membrane

Table 1 shows sodium thiopental concentration in the medium, after incubation with intact RBC or unsealed ghosts. A comparable amount of sodium thiopental was taken up from the medium by either structure, indicating that sodium thiopental is located in the cell membrane. Both desialated RBC and their ghosts did not differ in how much sodium thiopental they took up from the incubation medium, this value being significantly larger than that in their normal counterparts.

Effect of Sodium Thiopental on Erythrocyte Shape Changes

Thiopental is an anionically dissociated compound. Figure 1 illustrates, according to the Sheetz and Singer bilayer couple theory (4), how this amphipathic anion produces echinocytes with an index increasing when the drug concentration rises.

To verify whether the glycocalyx affected the interaction between the anesthetic and the membrane, RBC were incubated with neuraminidase that released 90% of the membrane sialic groups. Desialated erythrocytes suspended in thiopental solutions had a greater echinocyte index than the desialated control without sodium thiopental (Figure 1).

Erythrocyte Osmotic Fragility after Incubation with Sodium Thiopental

Osmotic fragility curves obtained with normal and sodium thiopental treated erythrocytes are shown in Figure 2. Sodium thiopental at 5 mM concentration diminished hemolysis, yielding significant differences (* p<0.05) at 0.35, 0.40

and 0.45 g/dl of NaCl.

Mechanical Properties of the Erythrocyte Membrane in the Presence of Sodium Thiopental

The presence of sodium thiopental in the RBC suspension caused an increase in cell deformability (Table 2).

Both μ and η were diminished by sodium thiopental; therefore, the treated cells membrane became more flexible and required a shorter time to recover the normal shape when the deforming force ceased (t_r was lower than in control ones).

The effect of sodium thiopental on erythrocyte membrane mechanical properties of neuraminidase treated cells is shown in Table 3.

Table 1. Sodium thiopental concentration (g/l) in the medium before and after incubation

	Before incubation	RBC	After incubation		
			RBCUG	N-RBC	N-RBCUG
Median	0.88 ^a	0.62 ^{a,b,c}	0.69 ^{a,d,c}	0.50 ^{a,b,e}	0.56 ^{a,d,e}
Range	0.60-1.42	0.36-1.10	0.34-0.90	0.30-0.90	0.28-0.73

RBC: red blood cell; RBCUG: red blood cell unsealed ghosts; N-RBC: neuraminidase treated red blood cells; N-RBCUG: neuraminidase treated unsealed ghosts. Data are presented as median and range (n: 7). Significance of the difference between medians sharing the same superscript: a, b and d: p< 0.05; c and e: > 0.05.

Table 2. Mechanical properties of the erythrocyte membrane with and without sodium thiopental. Mean \pm SEM (n: 14)

anesthetic	δ	t_r msec	$\mu \times 10^{-3}$ dynes / cm	$\eta \times 10^{-4}$ dynes / sec / cm
0 mM	0.42 \pm 0.008	114.10 \pm 6.44	4.15 \pm 0.09	4.76 \pm 0.33
5 mM	0.44 \pm 0.006	70.60 \pm 7.19	3.91 \pm 0.05	2.77 \pm 0.29
	p \leq 0.025	p \leq 0.001	p \leq 0.05	p \leq 0.001

Table 3. Mechanical properties of erythrocyte membrane of neuraminidase treated RBC with and without sodium thiopental. Mean \pm SEM (n :14)

anesthetic	δ	t_r msec	$\mu \times 10^{-3}$ dynes / cm	$\eta \times 10^{-4}$ dynes / sec / cm
0 mM	0.43 \pm 0.010	90.10 \pm 7.11	4.07 \pm 0.11	3.63 \pm 0.27
5 mM	0.46 \pm 0.012	149.88 \pm 11.42	3.79 \pm 0.12	5.72 \pm 0.49
	p \leq 0.001	p \leq 0.001	p \leq 0.001	p \leq 0.001

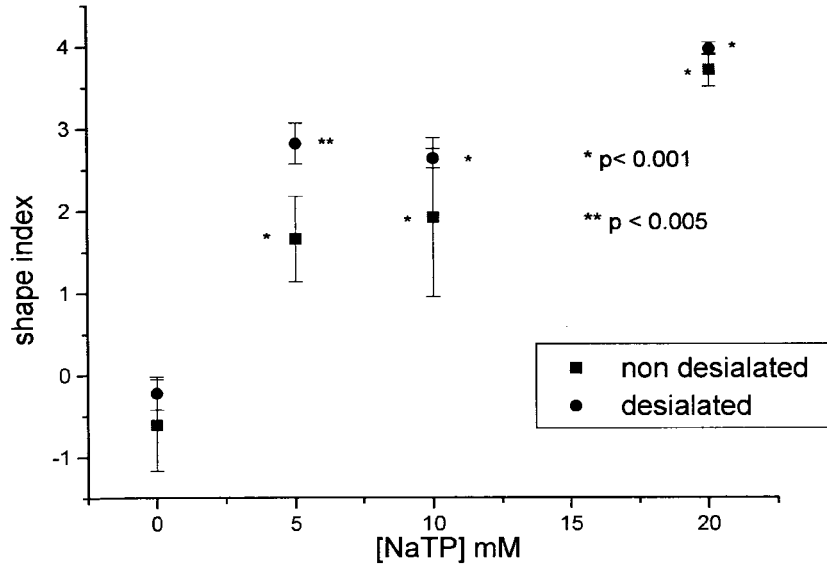


Figure 1. Shape index as a function of sodium thiopental (NaTP) concentration $\times \pm$ SD (n + 6)

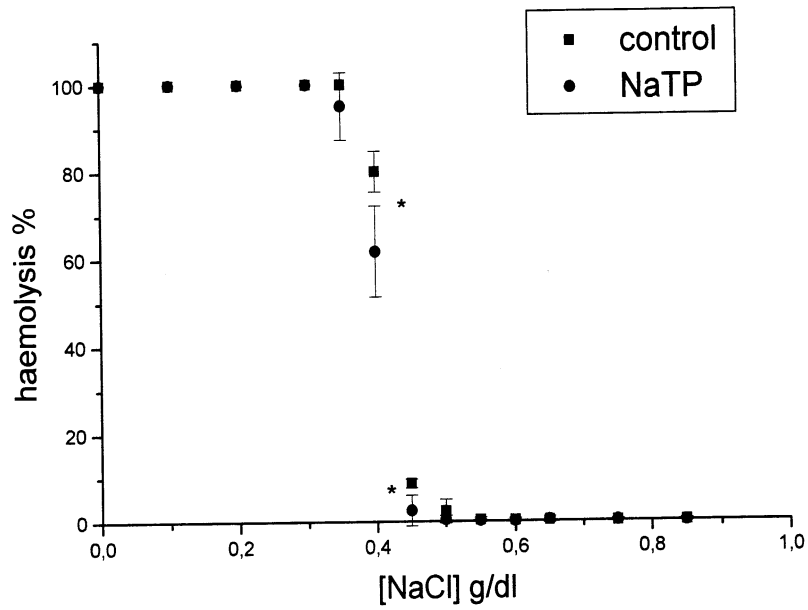


Figure 2. RBC osmotic fragility curves with and without sodium thiopental (NaTP) $\times \pm$ SD (n=6)

Table 3 shows that neuraminidase treated cells incubated with sodium thiopental, increased their deformability as did non desialated cells, though one of the parameters involved in determining deformability believe differently: η increased, at variance with nondesialated cells. Thus, desialated cells incubated with sodium thiopental were more deformable, needing a longer time to recover the initial shape when the shear force was interrupted (t_r was higher).

DISCUSSION

It is well known that membrane physical properties such as cell shape, hypotonic lysis resistance, permeability or cell aggregation, determined by the lipid bilayer, can be altered by the intercalation of amphipathic molecules (8). Mechanically, the bilayer is considered a two-dimensional fluid without elastic modulus and with viscosity two orders of magnitude lower than that of the whole membrane. Accordingly, membrane shear elasticity and viscosity are ascribed to the protein skeleton (1) with no participation of the bilayer.

Studies of the echinocytogenic agent's effect on blood cell deformability reported contradictory results. Reinhart *et al.* (18) observed that echinocytes produced by Na-salicylate were more filterable through 3 μm pores than discocytes; Leblond (19) found that echinocytes generated by oleate required the same filtration pressure as discocytes, whereas those produced by lysolecithin required less pressure than normal shaped erythrocytes. Bessis *et al.* (20), using lysolecithin and an ektacytometer, and Meiselman (21), by means of high-speed centrifugation and rheoscopy, demonstrated that echinocytes were less deformable than discocytes. Both the drugs and the methodologies employed might explain the described discrepancies, though the latter are not equally sensitive to factors determining erythrocyte mechanical properties. Chien (22) argues that deformability tests with geometric constraints, such as filtration through narrow pores, might be more influenced by cell

geometry than those without geometric constraints. The rheoscope, the ektacytometer and the apparatus used in our experiments fall into the latter category, in which the estimate of deformability mainly depends on the cytoplasm viscosity and the viscoelastic properties of the membrane.

The spectrophotometric measurements indicated that in our model the amphipath sodium thiopental was located in the erythrocyte membrane (Table 1). Moreover, the dose dependent echinocytogenic effect (Figure 1) confirmed its presence in the external hemilayer of the lipid bilayer (4) whereas the increased resistance to osmotic lysis (Figure 2) showed that, as other amphipathic agents, sodium thiopental expanded and fluidized the membrane. Likewise, treatment with sodium thiopental improved erythrocyte deformability, a parameter determined by cellular geometry, cytoplasm viscosity and membrane material properties. Since hemoglobin corpuscular concentration, a major determinant of cytoplasmic viscosity, remained constant throughout the experiments it can be assumed that cytoplasmic viscosity did not change; neither changed significantly the ratio surface area/volume. The effect of sodium thiopental on the elastic modulus and surface viscosity coefficient could then be attributed to modifications of the membrane mechanical properties (Table 2), specifically to the bilayer contribution to them. This bilayer involvement in the membrane mechanical properties might be either direct or through interactions between the bilayer and the cytoskeleton (23,24).

Spectrophotometric assays using desialated erythrocytes showed that sodium thiopental membrane insertion was higher than in intact ones (Table 1). Coincidentally, the echinocytic index produced by sodium thiopental was higher in desialated cells. Grebe *et al.* (25) reported a significant influence of the external surface charge on the erythrocyte membrane curvature. These authors observed, as we did in a previous study (26), that the charge reduction by enzymatic effect and the associated decrease in electrostatic

repulsion resulted in a stomatocytic shape. They also showed that this effect partially compensated that of the echinocytogenic agent sodium salicylate: the drug concentration had to be increased to attain an echinocytic index similar to that of the non-desialated cells. At variance, in the present work we observed that sodium thiopental induced a higher echinocytic index in desialated cells than in normal ones. These opposing results can be explained assuming that drug entrance into the lipid bilayer is increased in desialated cells. In the same way, Born *et al* (27) observed an increased access of chlorpromazine in desialated cells and postulated that the surface charge behaves as a charged barrier that hinders the drug entrance to the lipid bilayer.

From a rheological point of view, it should be noted that neuraminidase decreased membrane viscosity (30%), pointing out a key role for the glycocalyx and its internal frictional force on this parameter. Schmid-Schönbein *et al* (28) demonstrated that the electrostatic repulsion among the glycocalyx charged particles and the small-intercalated ions determines the resistance to changes in the membrane curvature. Likewise, the electrostatic repulsion might restrict the deformation produced by the shear stress so that charge removal from the glycocalyx would reduce the frictional force and diminish the erythrocyte surface viscosity. Both desialated and normal cells exhibited the same response to the anesthetic regarding the elastic modulus, but in an opposite direction regarding surface viscosity: it decreased in the normal cells and increased in the desialated ones. If removal of the charged barrier permits a higher drug concentration in the bilayer, it should be expected a membrane molecular disorganization manifested by an increase in the internal friction. This could also explain why the higher fragility of the desialated membranes precluded the assessment of the cell osmotic resistance when sodium thiopental was added to the medium.

Summarizing, our results allow us to postulate first, that sodium thiopental localized in the lipid bilayer can trigger modifications of the

membrane mechanical properties, showing the role of this structure in whole membrane rheology; secondly, that the glycocalyx negative charges are a steric and electrostatic barrier restricting sodium thiopental access to the membrane, so that desialation permits the entrance of most sodium thiopental molecules.

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