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Flunitrazepam partitioning into natural membranes increases surface curvature and alters cellular morphology

Daniel A. García [a], Santiago Quiroga [b], María A. Perillo [a,*]

[a] *Cátedra de Química Biológica, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, 5000 Córdoba, Argentina*

[b] *Dpto. de Química Biológica, CIQUIBIC (CONICET), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, CC 61, 5000 Córdoba, Argentina*

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Abstract

In recent studies, we showed that flunitrazepam (FNTZ) and other benzodiazepines interact with artificial phospholipid membranes locating at the polar head group region, inducing a membrane expansion, reducing the molecular packing and reorganising molecular dipoles. In the present paper we investigated the possibility that those phenomena could be transduced into changes in the curvature of membranes from natural origin. Hence we studied the effect of FNTZ on cellular morphology using human erythrocyte as a natural assay system. Shape changes of erythrocytes were evaluated by light microscopy and expressed as a morphological index (MI). FNTZ induced echinocytosis in a time-dependent manner with MI values significantly higher than those of control (without drug) or DMSO (vehicle) samples. Lidocaine, a local anesthetic known to induce stomatocytosis by incorporating in the inner monolayer, counterbalanced the concentration-dependent FNTZ crenating effects. FNTZ induced protective effects, compared with control and DMSO, against time-dependent hemolysis. Hypotonic-induced hemolysis, was also lowered by FNTZ in a concentration-dependent manner. Both antihemolytic effects suggested a drug-induced membrane expansion allowing a greater increase in cell volume before lysis. In such a complex system like a cell, curvature changes triggered by drug partitioning towards the plasma

Abbreviations: BZD, benzodiazepine; DMSO, dimethylsulphoxide; FNTZ, flunitrazepam; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MI, morphological index.

* Corresponding author. Fax: + 54-351-4332097.

E-mail address: mperillo@com.uncor.edu (M.A. Perillo).

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membrane, might be an indirect effect exerted through modifications of ionic-gradients or by affecting cytoskeleton–membrane linkage. In spite of that, the curvature changes can be interpreted as a mechanism suitable to relieve the tension generated initially by drug incorporation into the bilayer and may be the resultant of the dynamic interactions of many molecular fluxes leading to satisfy the spontaneous membrane curvature. © 2000 Published by Elsevier Science Ireland Ltd.

Keywords: Flunitrazepam; Benzodiazepine; Lidocaine; Erythrocyte; Membrane expansion; Anti-hemolytic effect

1. Introduction

Benzodiazepines (BZDs) are extensively used as anxiolytics, miorelaxants, anti-convulsants and hypnotics. Flunitrazepam (FNTZ) is a potent anxiolytic BZD; however, it has been mainly used as a night-time hypnotic and it has been applied to the induction of anesthesia in surgery. FNTZ has also been abused by opiate addicts for the relief of some of the signs of abstinence such as irritability and insomnia [1].

It is known that this kind of drug affects neurotransmission by interacting with biological membranes through specific membrane–integral proteins [2,3] and, due to their hydrophobicity, BZDs can also interact nonspecifically with a lipidic part of the membrane [4,5].

Previous reports from our laboratory demonstrated that nonspecific interactions of membrane–BZD could be explained by a partition equilibrium model (unlimited incorporation of molecules) [4]. The thermodynamic result of partition equilibrium is consistent with drug molecules accommodated between lipid molecules becoming an integral part of the membrane, inducing its swelling until it is converted to a non-bilayer phase [6].

In recent studies we showed that FNTZ and other BZDs interact with artificial phospholipid membranes locating at the polar head group region and expanding the membrane [7–9]. This interaction led to an increase of the relative volume of the polar head groups and to a decrease in the thermodynamic stability of the self-aggregating structure, which was forced towards an increase of its surface curvature. Molecular filtration [10] and electron microscopic [9] studies let us demonstrate that FNTZ is able to diminish the diameter of dipalmitoyl phosphatidylcholine (dpPC) vesicles. It was expected that membranes of more complex composition could show more responsive mechanisms. In this type of model, tension originated by the presence of the drug in the membrane should be compensated by passive fluxes of lipid molecules between both leaflets changing their lipidic compositions in order to satisfy their spontaneous curvature. Accordingly, in mixed liposomes composed of dpPC plus dipalmitoyl phosphatidylethanolamine (dpPE), FNTZ was able to induce a decrement in the amount of PE in the outer surface as a result of a molecular rearrangement between both leaflets in order to diminish the structural tension in the membrane originating from the drug incorporation [9].

The plasma membrane represents a supramolecular structure where not only the lipid bilayer and the associated proteins but also their interaction with the cytoskeleton take part in determining the surface curvature expressed as cell morphology. The modulation of curvature in a natural membrane may be achieved through changes in the physicochemical properties of the environment (e.g. pH gradients [11] as described in artificial membranes); moreover, changes in membrane curvature may be a consequence of modification in the cellular metabolic activity, affecting the phosphorylation state of membrane lipids and proteins which in turn participate as links with the cytoskeletal network [12].

Human erythrocytes, having no internal organelles, have been used as appropriate cellular models to study the drug–biomembrane interaction [13,14]. It has already been well established that mature red blood cells undergo one of two types of membrane transformation, and a resultant shape change of the cells under in vitro action of amphipathic compounds. According to the bilayer-couple hypothesis of Sheetz and Singer [15], neutral or negatively charged amphipaths produce the transformation from discoid normal cells to evaginated echinocytes by their preferential association with the outer monolayer, probably due to the inability of this type of amphipaths to cross the bilayer or because of electrostatic repulsion between negatively-charged head group of lipids in the inner monolayer. Conversely, positively-charged amphipaths generate invaginated stomatocytes perhaps by interaction with the negatively-charged phospholipids [16,17]. The major lipids composing the erythrocyte membrane are distributed asymmetrically across the bilayer [18,19]; while PC and sphingomyeline are found predominantly in the outer monolayer, negative aminophospholipids (phosphatidylserine and PE) are located in the inner monolayer [13,20]. Lipid asymmetry is considered to be mainly maintained by an ATP-dependent aminophospholipid translocase that transports this kind of lipids from the outer to the inner monolayer [13,17,21]. It was proposed that the treatment of erythrocytes with amphipathic drugs was able to induce flippase-mediated selective translocation of the aminophospholipids [22]. In this sense, Chen and Huestis [13] demonstrated that chlorpromazine induced a movement of phosphatidylserine from the inner to outer monolayer. Recently, compensating passive lipid fluxes to the exoplasmic leaflet have been described, counterbalancing tensions originated on the active transport of aminophospholipids to the cytoplasmic monolayer by the aminophospholipid translocase [23].

The purpose of the present work was to investigate if the ability of FNTZ to affect the curvature of artificial membranes could be exerted in natural membranes and transduced to changes in cellular morphology. Therefore, we used human erythrocytes as a natural assay system.

2. Materials and methods

2.1. Materials

FNTZ (7-nitro-1,3-dihydro-1-methyl-5-(2-fluorophenyl)-1,4-benzodiazepin-2-one)

was kindly supplied by Hoffman La Roche (Basle, Switzerland). Dimethylsulphoxide (DMSO), glutaraldehyde and lidocaine were obtained from ICN Pharmaceutical (Costa Mesa, CA). All other chemicals were of analytical grade.

2.2. Erythrocyte preparation

Human blood was obtained from healthy volunteers by venipuncture and collected into sodium citrate 3.8% (w/v) as anticoagulant (blood/citrate ratio, 10:1). Erythrocytes were separated by centrifugation ($200 \times g$ for 5 min), and plasma and buffy coat removed by aspiration. They were then washed two times with 2 vol. 150 mM NaCl and once with 2 vol. buffer A: 138 mM NaCl, 5 mM KCl, 6 mM Na_2HPO_4, 1.5 mM NaH_2PO_4 and 5 mM glucose, pH 7.4 (NaCl/Pi).

2.3. Treatment of erythrocytes with drugs

Fresh erythrocyte suspensions were used at a final hematocrit of 3–4%. FNTZ and lidocaine were added from stock solutions prepared daily in 150 mM NaCl. All samples except control without drugs (which had only 150 mM NaCl) contained 1% DMSO final concentration. Samples were incubated at room temperature for 5 min to 19 h, depending on the experiment.

2.4. Cell morphology

Samples of 0.1 ml of each erythrocyte suspension were prepared for morphological analysis by adding 0.4 ml of 0.5% glutaraldehyde in 150 mM NaCl for at least 15 min. Small aliquots of the prepared samples were observed with a light microscope equipped with differential interference contrast using a $63\times$ objective. Some fields were randomly chosen and the images were captured using a Princeton Micromax cooled CCD camera and digitized directly into a Metamorph Image Processor. Photographs were printed using Adobe Photoshop for analysis.

A minimum of 80 cells were analysed and scored for each sample. A normal biconcave disc shape was given a score of zero. Echinocytes and stomatocytes were given scores of $+1$ to $+4$ (externalization) or -1 to -4 (internalization) respectively according to Fujii et al. [16]. Examples of typical morphologic cellular types are shown in Fig. 1. The morphological index (MI) was calculated as the sum of the products of the scores corresponding to a determined shape stage of cells, times the total cell number in the corresponding stage.

2.5. Determination of hemolysis

After incubation, cell suspensions were centrifuged at $1500 \times g$ for 20 min. The hemoglobin content of the supernatants was estimated from absorbance at 540 nm

with a Beckman DU 7500 spectrophotometer. Hemolysis was calculated as the amount of hemoglobin released, normalized by division with the value of the control sample at 5 min (relative hemolysis).

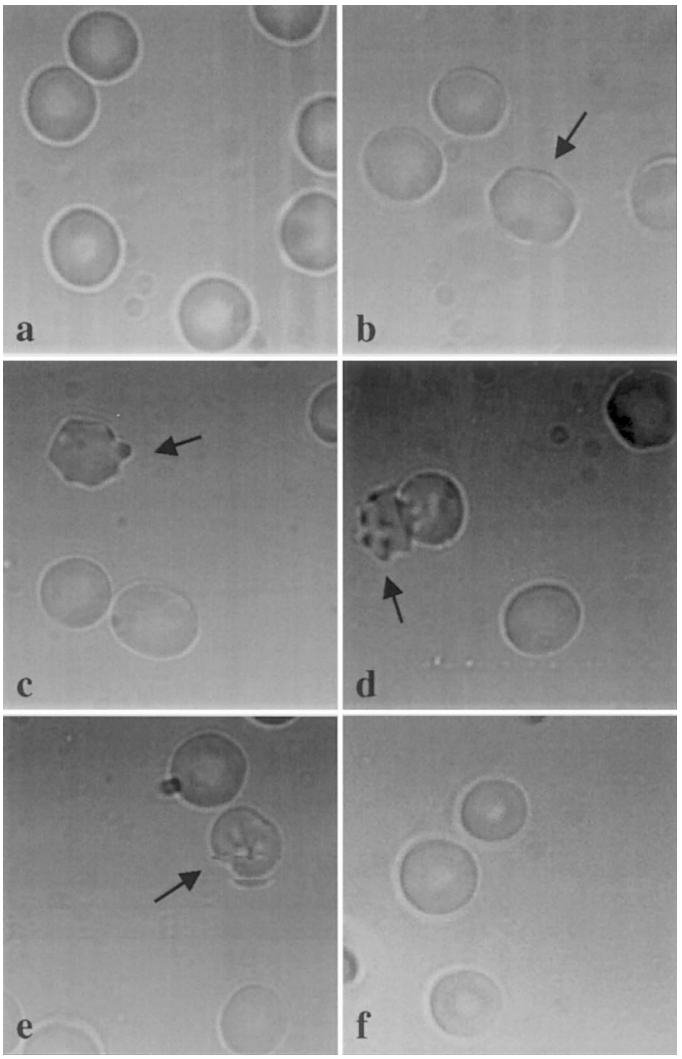

Fig. 1. Typical examples of the different erythrocyte shapes found. The erythrocyte suspensions were incubated in the presence of: buffer A for 5 min (a) or 100 μM FNTZ for 30 min (b), 120 min (c) and 19 h (d); 500 μM lidocaine for 120 min (e) and 100 μM FNTZ plus 750 μM lidocaine during 120 min (f). The arrows indicate typical morphologies corresponding to echinocytes in different stages (b,c,d) and a stomatocyte (e). In panels a and f, all erythrocytes exhibit the normal discocyte morphology.

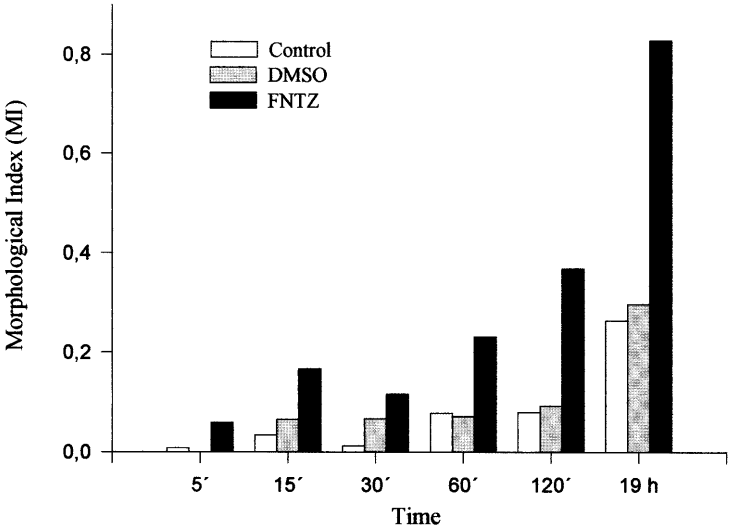

Fig. 2. Effect of FNTZ on erythrocyte morphology. The morphological index at different incubation times was calculated as indicated in Section 2. The cell suspension contained buffer A (Control), 1% DMSO (DMSO) or 100 μM FNTZ dissolved in 1% DMSO (final concentrations) (FNTZ), respectively. All samples were incubated at room temperature.

2.6. Determination of anti-hemolytic effect

Washed erythrocytes were suspended at 0.8% final hematocrit in 75 mM NaCl for 5 min at room temperature. This NaCl solution corresponded to a hypotonic medium capable of producing approximately 30% of absolute hemolysis. The hypotonic solution contained FNTZ (10 nM to 1 mM) dissolved in DMSO or DMSO at the same final concentrations reached in the corresponding FNTZ containing sample (2.5×10^{-5} to 2.5%, v/v). The hemoglobin content was determined as indicated above.

3. Results

The cell shape changes, like those shown in Fig. 1, were graphically represented by scoring representative samples on a MI scale. Fig. 2 shows the time course of erythrocyte shape changes expressed as the MI in the absence or presence of 100 μM FNTZ. This drug induced echinocytosis in a time-dependent manner. After 5 min of exposure to FNTZ, normal discoid erythrocytes became echinocytic and the MI increased with larger incubation times, except from 15 to 30 min where the MI decreased slightly. The echinocytosis also increased with incubation time in control and DMSO samples. Both control and DMSO samples exhibited similar values of MI between them, but significantly lower than those obtained with FNTZ.

In order to investigate if those cell-shape changes were mediated by membrane expansion, FNTZ effects on hemolysis in isotonic medium and FNTZ effects on hypotonic-induced hemolysis were studied [24]. Fig. 3 shows the time course of the anti-hemolytic effects induced by FNTZ in isotonic medium. Up to 120 min of incubation, the different samples did not show significant levels of hemolysis. At 19 h, in control and DMSO samples, the relative hemolysis increased 26 and 20 times, respectively compared to the control at 5 min; with FNTZ a significantly lower degree of hemolysis compared with control and DMSO was observed (note that in this sample hemolysis was only 14 times higher than the control at 5 min). The effects of increasing concentrations of FNTZ and DMSO on the relative hypotonic hemolysis of erythrocytes are shown in Fig. 4. In this case, the relative hemolysis corresponds to the hemolysis measured in respect to control in hypotonic medium containing neither FNTZ nor DMSO. FNTZ displayed an anti-hemolytic effect with respect to the controls. This protection increased slightly up to 100 μM while, at 1 mM FNTZ, the degree of hemolysis was quite similar to that of the control. DMSO slightly increased the hemolysis with respect to the controls and at 2.5% of DMSO the hemolysis increased more abruptly. The difference between both experimental curves gave the net decrement in relative hemolysis induced by FNTZ, with respect to the normal value of 1.

Other proofs in favour of the hypothesis that FNTZ effects on cell-shape were exerted at the membrane level were obtained by measuring the action of this BZD in the presence of another drug whose effects on red blood cells were known to (1)

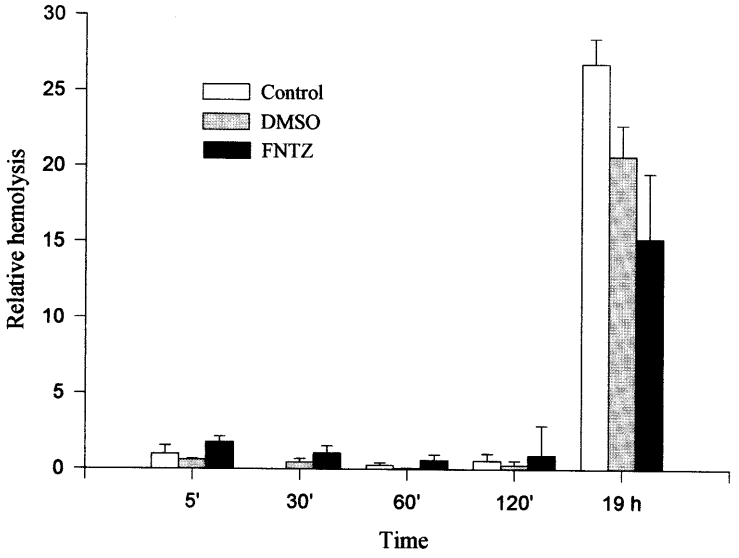

Fig. 3. Effect of FNTZ on erythrocyte hemolysis. The relative hemolysis, calculated as described in Section 2, was measured as a function of the incubation time. The hemolysis in control sample at 5 min was given a value of 1. The samples Control, DMSO and FNTZ correspond to the same conditions indicated in Fig. 1. The values represent the average of triplicates and error bars are S.E.

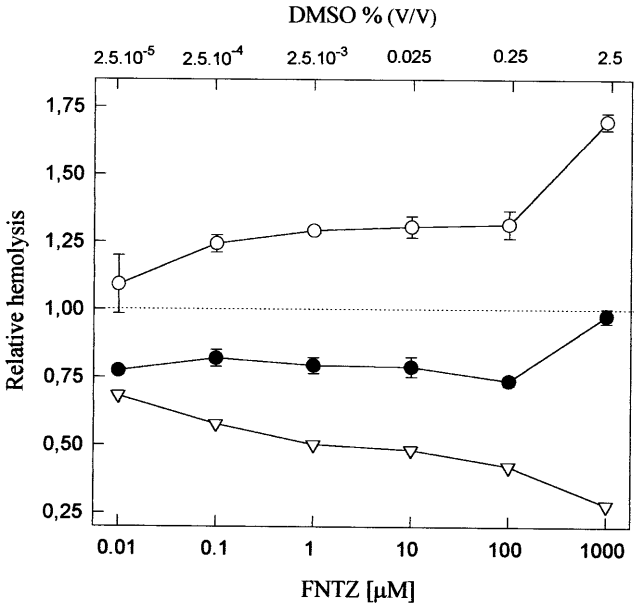

Fig. 4. Effect of FNTZ on hypotonic hemolysis of erythrocytes. The hemolysis, expressed as relative hemolysis, was measured in the presence of FNTZ (●) or DMSO (○) at several concentrations. The differences between both experimental curves represented the net FNTZ effect (▽). A value of 1 (dotted line) was given to the relative hemolysis in the control sample (see Section 2). The values of DMSO, indicated in the top axis, are the final concentrations equivalent to those added as solvent in FNTZ samples. The incubation time was 5 min. The values represent the average of triplicates and error bars correspond to S.E.

be exerted through its membrane incorporation and (2) be in the opposing direction with respect to that found for FNTZ. Lidocaine (500 μM) induced stomatocytocis in the red blood cells (MI = −0.34) (Fig. 5). FNTZ at a concentration of 2 μM was able to reverse these stomatocytes to a normal discoid shape. Higher concentrations of FNTZ induced increasing echinocytosis. Conversely, the presence of 100 μM FNTZ produced a positive value of MI (0.6) corresponding to echinocytosis and increasing concentrations of lidocaine in this system reduced the degree of echinocytosis up to 1000 μM lidocaine where the reversal of MI towards negative values started (stomatocytocis).

4. Discussion

The mechanism of amphipath-induced shape changes in red blood cells has been under investigation for a long time. Many works have demonstrated that negatively-charged or neutral amphipaths induced echinocytosis by their intercalation into the outer monolayer of the erythrocyte membrane, resulting in net outer monolayer expansion and crenation [16,17] according to the bilayer-couple hypoth-

esis of Sheetz and Singer [15]. The ability of benzodiazepines like FNTZ to partition from an aqueous media towards a membrane either artificial [7–10,25] or natural [5] including erythrocytes [26], was stated using several methodologies like monomolecular layers at the air–water interface, radioligand binding, spectroscopic fluorescence and electron spin resonance. The membrane/buffer partition coefficient for FNTZ had a value around 20 in both types of membranes and although affected by pH, temperature, saline concentration and cholesterol content, it remained within the same order of magnitude [5,25]. So, a similar value should be expected for the erythrocyte–buffer FNTZ partition coefficient.

This drug would behave as a substitional impurity as suggested by its incorporation in the polar head group region of membranes promoting membrane expansion, increasing the curvature and the translocation of phospholipids [7–9,25].

In the present work, FNTZ induced a higher degree of echinocytosis with respect to control samples at all the times assayed (Fig. 2). It was reported for many shape-transforming compounds that the echinocyte formation was accompanied by an increase in the outer surface area [27]. FNTZ ($pK = 1.5$ [7]) was neutral in the incubation media used (pH 7.4) and, according to the classical bilayer-couple hypothesis [15], could contribute to an unequal surface area of the two leaflets of the bilayer expanding the outer monolayer and producing echinocytes. This fact suggested that, membrane expansion could be exerted by FNTZ not only in model membranes [9] but also in natural ones. Superimposed to the FNTZ effect, we

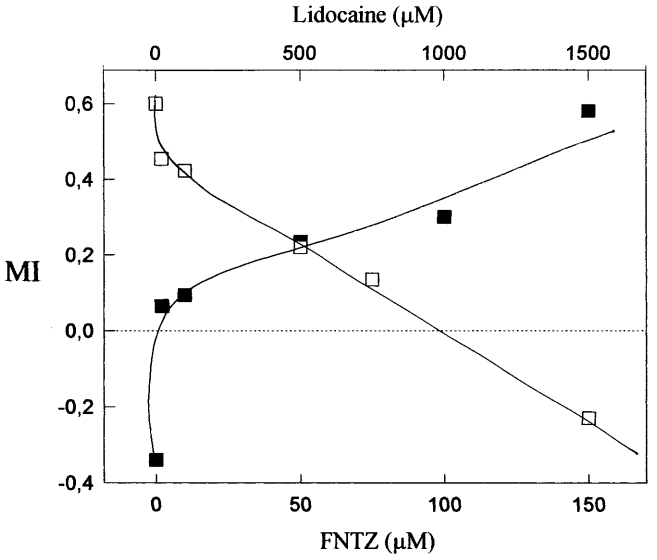

Fig. 5. Compensating effects between FNTZ and lidocaine on erythrocyte morphology. Erythrocytes were incubated at room temperature for 120 min with: 100 μM FNTZ in the presence of lidocaine at different concentrations (0–1.5 mM) (□) or 500 μM lidocaine in the presence of FNTZ at different concentrations (0–150 μM) (■). The MI value of 0 corresponds to normal shape cells and is indicated with a dotted line.

observed a general trend of Control, DMSO and FNTZ samples to increase MI with time. This is possibly related to the impairment, due to substrate consumption, of energy requiring processes involved in the maintenance of concave shape. At short time periods, those postulated mechanisms might still be functional allowing a partial compensation of FNTZ effects at 30 min (Fig. 2).

The increment of MI at the longest incubation periods, in all the conditions assayed, might be explained by a possible depletion of ATP sources which caused a strongly marked echinocytosis leading to two possible main consequences: (1) alterations in phosphoinositide metabolism that could have led to a movement of metabolites from the inner to the outer half of the lipid bilayer, resulting in echinocytosis [28] and (2) a deficiency in the compensating mechanism associated with the activation of the ATP-dependent aminophospholipid translocase which, after the expansion of the outer or the inner monolayer induced by drug incorporation, was unable to restore the normal biconcave shape of cells by transport aminophospholipids from the outer to inner monolayer [19,23]. This hypothesis may be supported by the fact that the highest degree of echinocytosis, corresponding to 19 h of incubation, coincided with an abrupt increment in the hemolysis, in all the conditions assayed (Fig. 3). At this specific point, the samples incubated with FNTZ showed a hemolysis significantly lower than that of control. Perhaps, for FNTZ-containing samples, the high degree of crenation observed at the longest time assayed was promoted by the two mechanisms indicated above and, in addition, by an expansion of the outer monolayer induced by the drug incorporation. According to Motais et al. [24], the phenomenon of membrane expansion can be quantified by measuring the degree of protection from hypotonic hemolysis in the presence of different concentrations of compounds capable of incorporation in the membrane; the hemolytic protection is interpreted as being due to an expansion of the membrane allowing greater increases in cell volume before lysis. Following this rationale and in agreement with previous reports for other BZDs [26,29,30] our results demonstrated that FNTZ had an apparent anti-hemolytic effect. This effect was maximal at the assayed FNTZ concentration of 0.1 mM and, at 1 mM, the hemolysis apparently increased (Fig. 4). In spite of the fact that this apparently maximal anti-hemolytic concentration of FNTZ was of the same magnitude as those previously reported for diazepam (0.2 mM [29] and 0.9 mM [30]), the increment in the hemolysis at higher FNTZ concentrations coincided with a marked increase in hemolysis in the presence of DMSO at the same concentration present in FNTZ containing samples. This result, in addition to the fact that the previous studies did not state clearly the concentration of the solvent used as BZD vehicle, suggest that the maximal anti-hemolytic concentration of BZDs would have been even superior. This became evident when, assuming that mixed DMSO + FNTZ effects were the addition of the individual effects of both drugs administered separately, we determined, from the difference between DMSO and FNTZ + DMSO samples, a net concentration-dependent FNTZ-induced decrement in relative hemolysis with respect to the normal value of 1 (Fig. 4).

Lidocaine is a local anesthetic that belongs to the group of amphipath amines. This kind of drug interacts with erythrocyte membranes inducing internalization or

stomatocytosis by their incorporation in the inner monolayer [16,31]. This anesthetic, incubated simultaneously with FNTZ, produced a cell shape change that depended on the relative proportion of each drug in the system (Fig. 5). It is well established that when a crenator drug and an invaginator drug are added simultaneously to an erythrocyte suspension, they can be incorporated independently into separate monolayers and to exert an antagonistic effect as a result [16]. Thus, the addition of both drugs at the appropriate concentrations induced the apparition of normal shape cells, in spite of the presence of the drugs in the membrane. The reason why the extracellular concentration of the crenator and the invaginator required to give antagonistic effects were not equal, might have been due to the differences between their membrane–aqueous phase partition coefficients, the type of short-range interactions that they established with individual membrane components and the coherent effects that they are able to exert in the supramolecular assembly of the membrane.

5. Possible mechanism of FNTZ-induced erythrocyte shape change

5.1. Membrane curvature

Packing properties of molecules in bilayers and non-bilayers structures depend on thermodynamic factors coupled to molecular geometry [32,33]. Steric restrictions are imposed on the interfacial spontaneous curvature derived from the complementary shape of the molecules in a self-assembled structure. This molecular dynamics may be relevant in a wide spectrum of biological phenomena involving curvature changes that participate in many cellular processes mediated by membrane interactions, recombination and signalling [11,34–36] which might be associated with the non-receptor mediated BZD effects [8].

The intensity required for a perturbation to be able to destabilize the structure will depend on the membrane elastic compressibility. In this sense, proteins may play an important role in softening the dynamic response and determining a difference between the behaviour of liposomes and proteoliposomes (e.g. natural membranes). Even though, our results suggest that not only in model membranes [9,10] but also in natural membranes (present paper) the non-receptor mediated interaction with FNTZ may induce an increase in the outer monolayer curvature and evaginations. Such curvature changes can be interpreted as a mechanism suitable to relieve the tension generated by drug incorporation into the bilayer.

Finally, the morphology of cells is the resultant of the dynamic, and possibly non-linear, interaction of many molecular fluxes. According to the thermodynamics of open systems far from equilibrium, perturbations on those fluxes (e.g. molecular movements from one monolayer to the other) and/or on their conjugated forces (e.g. curvature tensions), can modify the original stationary state of the system. This leads the system to be stabilized in a new stationary state represented by a different cellular shape.

5.2. Cytoskeleton organisation, ionic pumps and environmental variables

The molecular dynamics within the membrane may be affected by environmental changes in pH, ionic strength, polymerization–depolymerization equilibria of cytoskeletal proteins, etc. The actin-containing vesicles have demonstrated that polymerizing actin can deform the vesicle-membranes. However, this qualitative observation does not immediately mean that the growing actin filaments can push the cell membranes, because the bending moduli of the cell membranes are 5–50 times larger than those of the vesicle-membranes [37,38]. It was reported that the administration of diazepam, another BZD, in vivo induced an organisation of microfilaments in macrophages [39]. Diazepam was also described as an anti-microtubule agent in diatoms [40]. However, in none of these articles were cellular shape changes reported. Moreover, while the effects in the former report were observed after a long chronic treatment, in the latter the concentrations used were so high that huge amounts of a hydrophobic vehicle (not indicated) must have been required.

Any asymmetry in molecular species and/or other fields across the bilayer will result in a curved equilibrium configuration of the membrane. Asymmetry may be created by surface fields, like amphiphile monolayer densities and charge and steric configuration of adsorbed molecules, as well as boundary values of bulk fields at the interphase, such as electrolyte concentration and solution pH [11,41,42].

Changes in environmental variables can be triggered by changes in membrane properties, e.g. a change in membrane organisation can modify the activity of membrane enzymes and channels, leading to changes in ionic gradients across the membrane which in turn will have feedback effects on membrane organisation as well as in cellular function. High diazepam concentrations were found to inhibit calmoduline-dependent (Ca^{2+}, Mg^{2+})ATPase leading to accelerated erythrocyte destruction [43]. This is also the result of a chronic treatment and may be related to changes in erythrocyte lipidic composition [44]. These results are opposed to the antihemolytic effects of FNTZ found by us (Figs. 3 and 4) and by others [26,29,30] after acute treatments.

6. Conclusions

In such a complex scenario it cannot be unequivocally stated which was the exact pathway followed by erythrocytes after the initial FNTZ–membrane interaction towards the shape change. However, the concentration-dependent mutual compensating effects of FNTZ and lidocaine on erythrocyte shape in addition to the proven ability of membrane-binding drugs (including lidocaine) to induce phospholipid translocation [13,31] supports the idea of a direct effect of FNTZ on membrane curvature although, at present, other indirect mechanisms cannot be discarded.

The present results may contribute to study the pharmacological effects of BZDs from a dynamical perspective.

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