Ca$^{2+}$ Enhancement of Hemolysis Induced by the Topical Anesthetic Oxethazine in Vitro

Ryo-ichi Yasuno, Tamami Oguma, and Yasusuke Masuda

Division of Toxicology, Niigata College of Pharmacy, 5–13–2 Kamishinō-cho, Niigata 950–2081, Japan.
Received May 11, 1998; accepted September 2, 1998

Oxethazine (OXZ), a potent topical anesthetic, was found to induce red blood cell (RBC) lysis in vitro, depending upon concentrations of OXZ, RBC and Ca$^{2+}$. In a 2% RBC suspension, 100 $\mu$M OXZ caused almost complete hemolysis in the presence of 1.3 mM Ca$^{2+}$ with only a minimal effect in its absence, while higher concentrations of OXZ (400 $\mu$M) produced hemolysis without Ca$^{2+}$. The hemolysis induced by OXZ plus Ca$^{2+}$ was preceded by a rapid increase in $^{45}$Ca$^{2+}$ uptake by RBCs, with both the hemolysis and Ca$^{2+}$ uptake being inhibited by 1 mM CoCl$_2$, NiCl$_2$, and quinine. Together with the Ca$^{2+}$ influx, rapid influx of Na$^+$ and efflux of K$^+$ occurred, and an increasing external K$^+$/Na$^+$ concentration ratio inhibited both hemolysis and Ca$^{2+}$ influx. Morphologically, OXZ plus Ca$^{2+}$ caused rapid transformation to spherocytocytes, the formation of blebs and the pinching-off of blebs, whereas OXZ alone produced membrane invagination. SDS-PAGE analysis of the ghosts prepared from the RBCs treated with OXZ plus Ca$^{2+}$ revealed derangement of cytoskeletal components. OXZ is a rare drug that exhibits a Ca$^{2+}$ ionophore-like action, increasing the Ca$^{2+}$ permeability of plasma membranes.

Key words: oxethazine; Ca$^{2+}$ influx; erythrocyte; hemolysis

Oxethazine (OXZ) has long been prescribed clinically for esophageal, chronic gastritis and peptic ulcers, because of its potent topical anesthetic action, even at low gastric pH, and inhibitory action on gastrin secretion. From toxicity data, this drug appears relatively safe when given orally but highly toxic on i.v. administration. Aside from this, relatively little is known about the biological actions of OXZ despite its wide use. Chemically, OXZ as a weak base is relatively unionized in acidic solutions, whereas its hydrochloride salt is fairly soluble in organic solvents and, thus, it can penetrate cell membranes. In our previous study in an isolated rat liver perfusion system, infusion of 10 $\mu$M OXZ markedly increased portal perfusion pressure accompanying an inhibition of oxygen uptake, but only when Ca$^{2+}$ was present in the perfusate. This OXZ action is novel, not being shared by other local anesthetics. OXZ also produced contraction of isolated portal vein preparations, and, more recently, the inhibition of oxygen uptake was shown to result from uneven perfusion of the liver, due to contraction of peripheral portal vein branches. We hypothesized that OXZ could increase the Ca$^{2+}$ permeability of plasma membranes to produce contraction of the smooth muscle cells of the portal vein. To test this possibility, we selected red blood cells (RBCs) as a model membrane system.

In RBCs, the calcium ionophore A-23187 has been demonstrated to induce Ca$^{2+}$-dependent biochemical and morphological plasma membrane alterations. Although a variety of amphiphilic drugs including some tranquilizers, antihistamines and local anesthetics are known to have membrane-stabilizing action at low concentrations, preventing the hypotonic hemolysis of RBCs in vitro, they usually induce hemolysis at higher concentrations as exemplified by chlorpromazine. However, the Ca$^{2+}$-dependence of drug-induced hemolysis has rarely been reported. In the present study, we have shown that OXZ markedly enhanced hemolysis by increasing Ca$^{2+}$ permeability of RBC membranes, in conjunction with morphological and cytoskeletal changes.

MATERIALS AND METHODS

Chemicals OXZ (Sigma Chemical Co., St. Louis, MO) was dissolved in an equimolar HCl solution at a concentration of 10 mM, pH 3.5. $^{45}$Ca (specific activity, 26.11 mCi/mg) was purchased from E. I. Dupont de Nemours & Co., Inc., Boston, MA. Other reagents were of analytical quality and commercially available.

Preparation of RBCs RBCs from male, SPF-grade Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) weighing between 200–300 g were used. The animals were housed in an air-conditioned animal room (temperature, 23 ± 1°C, humidity 50–60%), and supplied with food and water ad lib. Blood was obtained by cardiac puncture under pentobarbital anesthesia (50 mg/kg, i.p.) using a heparinized syringe. RBCs were washed 4 times with about a 10-fold volume of modified Ca$^{2+}$-free Krebs-Henseleit buffer (KH-Tris buffer, 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 5.6 mM glucose and 20 mM Tris–HCl, pH 7.4), and finally suspended in this buffer to give a hematocrit value of 20%. For studies of cytoskeletal changes, RBCs from healthy human volunteers were used.

Incubation Conditions and Measurement of Hemolysis The incubation mixture contained 2% RBCs in KH-Tris buffer, with or without Ca$^{2+}$ and OXZ, in a final volume of 2 or 5 ml. OXZ was added last and the reaction was performed at room temperature (20–25°C) unless otherwise noted. At appropriate times, an aliquot was centrifuged using a microcentrifuge, the supernatant diluted and the optical absorbance read at 545 nm. Hemolytic indices were expressed as a percent of the complete hemolysis in water. Experiments were done in duplicate.

$^{45}$Ca$^{2+}$ Uptake and Na$^+$ and K$^+$ Contents For determination of $^{45}$Ca$^{2+}$ uptake, RBCs (2%) were incubated with various concentrations of Ca$^{2+}$ and OXZ with 1 $\mu$Ci $^{45}$Ca$^{2+}$ in a final volume of 1 ml. At appropriate times, a 0.2 ml aliquot was layered on top of 1 ml dibutyl phthalate in a 1.5 ml sample tube and centrifuged at 5000 g for 20 s using a...
microcentrifuge with a swing rotor (Kubota, type 1120). The top aqueous layer was removed and the tube washed several times with water down to the bottom RBC layer, to which 0.1 ml Protosol (New England Nucaer), 0.1 ml 3% H2O2, and then 0.8 ml Aquasol-2 (New England Nucaer) were added, in this order, after each mixing. The radioactivity was measured in a Liquid Scintillation Analyzer, Model 2200CA (Packard).

For cellular Na+ and K+ assays, a 10% RBC suspension was incubated with 400 µM OXZ plus 1.3 mm Ca2+ or with 800 µM OXZ alone in a final volume of 10 mL. At appropriate times, RBCs in a 2 mL aliquot were separated by centrifuging through a layer of dibutyl phthalate. The RBCs were treated with 4 mL 1 N HNO3 for 1 h, centrifuged at 10000 g for 1 h, and the supernatant was assayed for Na+ and K+ by flame photometry after dilution with 0.1 N HNO3.

Data are expressed as means±S.D. Statistical analysis was performed by Student's t-test and p<0.05 was considered statistically significant.

**Scanning Electron Microscopy** Aliquots of the regular reaction mixture were fixed in 1% glutaraldehyde, dehydrated, dried below the critical point, coated with gold and examined by electron microscopy (JEM-100CX, JEOL Ltd., Tokyo, Japan).

**Ghost Preparation and Electrophoretic Analysis** The method of Fairbanks et al.13 was used. Human RBCs (2%) were incubated for 30 min in 80 mL KH-Tris buffer in the presence and absence of OXZ and Ca2+. The incubation mixture of the hemolyzed groups, i.e., 150 µM OXZ plus 1.3 mm OXZ, and 500 µM OXZ alone (hemolysis; 95.5 and 96.0, respectively) were centrifuged at 3000 g for 20 min to obtain the ghost fraction. For control conditions, i.e., without or with 1.3 mm Ca2+ alone, and 150 µM OXZ alone (hemolysis; 1.3, 1.3 and 5.0%), RBCs were separated at 1500 g for 5 min and disrupted in 10 mm Tris–HCl (pH 7.9, washing buffer), then the ghost fraction was sedimented as above. Each ghost fraction was washed once with 40 mL cold 0.15 M KCl–1 mm EDTA–20 mm Tris–HCl (pH 7.4) and 4 times with the washing buffer by centrifuging at 10000 g for 20 min. Protein content of the ghost preparation was determined by Lowry's method.14 SDS–PAGE analysis was conducted according to the method of Laemml,15 using 3–15% gradient gel. Ghosts were solubilized in the presence of 2.5% SDS, and 20 µg protein per well was charged.

**RESULTS**

**General Features of the Hemolysis Induced by OXZ** As shown in Fig. 1A, hemolysis was induced by incubation of rat RBCs with OXZ for 30 min at 25°C in regular KH-Tris buffer containing 1.3 mm Ca2+, the physiological plasma Ca2+ concentration. The degree of hemolysis was dependent on both RBC and OXZ concentrations, but more than 60 µM OXZ was necessary to induce hemolysis at the lowest RBC concentration of 0.5%. In the time-course study with 2% RBC, 100 µM OXZ produced about 95% hemolysis in 30 min (Fig. 1B). The hemolysis induced by 100 µM OXZ was also dependent on the Ca2+ concentration (Fig. 1C); the effect was near maximum at 1.3 mm Ca2+ and was minimal in the absence of added Ca2+. With no added Ca2+, 400 µM OXZ produced near maximum hemolysis at 30 min (Fig. 1D). The time-course of hemolysis was almost unaffected by the incubation temperature between 10–37°C. In the following experiments, Ca2+-dependent and independent hemolysis was regularly induced by 100 µM OXZ plus 1.3 mm Ca2+ and 400 µM OXZ without Ca2+, respectively, in 2% RBC suspension at a temperature of 20–25°C.

**45Ca2+ Uptake** These experiments were done before marked hemolysis developed, i.e., the degree of hemolysis being under a limit of 20% as judged from the data in Fig. 1. In Fig. 2, calcium uptake was calculated by assuming that the 45Ca2+ uptake is due to a net Ca2+ movement into RBCs, and the calculated cellular Ca2+ uptake is not corrected for hemolysis since the mechanism of hemoglobin release is still unidentified. As Fig. 2A shows, Ca2+ uptake by RBCs in the presence of 1.3 mm Ca2+ was markedly increased by increasing the concentration of OXZ; with 100 µM OXZ, Ca2+ uptake was very rapid and amounted to 750 nmol/ml RBCs by 5 min. This uptake is quite large considering an intracellular ionized Ca2+ concentration of less than 0.1 µM and a total cell calcium of 5–15 mmol/ml in human RBCs.16,17 The

![Fig. 1. Some General Profiles of RBC Lysis Induced by OXZ in Vitro](image_url)

(A) Dependence of hemolysis on RBC vs. OXZ concentrations. Various concentrations of rat RBCs (0.5–10%) were incubated with 0–200 µM OXZ in KH-Tris (pH 7.4) containing 1.3 mm Ca2+ at 25°C for 30 min. Degrees of hemolysis are expressed as a percent of the complete hemolysis for each RBC concentration. (B) Time-course of the hemolysis induced by various concentrations of OXZ (0–200 µM), with 2% RBC and 1.3 mm Ca2+. Dotted line: without OXZ. (C) Time-course of the hemolysis induced by various concentrations of Ca2+ (0–2.6 mm), with 100 µM OXZ and 2% RBC. Dotted line: without Ca2+. (D) Comparison of the hemolysis induced by different concentrations of OXZ in the presence and absence of 1.3 mm Ca2+. Measured at 30 min with 2% RBCs. Values are means±S.D. (n=4–5).
Fig. 2. Enhancement of Ca\(^{2+}\) Uptake by OXZ
(A) RBC suspension (2%) was incubated with 0—100 \(\mu M\) OXZ in the presence of 1.3 mM Ca\(^{2+}\) with 1 \(\mu M\) \(^{65}\)Ca\(^{2+}\), and RBCs were separated at the times indicated.
(B) Ca\(^{2+}\) concentration dependence in the presence of 100 \(\mu M\) OXZ. The calculated cellular Ca\(^{2+}\) uptake values are not correct for hemolysis occurring during incubation. See the Materials and methods section for details. Values are mean ± S.D. (n = 4—5).

Table 1. Effects of Co\(^{2+}\), Ni\(^{2+}\), Mn\(^{2+}\) and Quinine on the RBC Lysis and Ca\(^{2+}\) Uptake Induced by OXZ

<table>
<thead>
<tr>
<th>Additions</th>
<th>Hemolysis (%) at 30 min</th>
<th>Ca(^{2+}) uptake (nmol/ml RBCs) at 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without OXZ)</td>
<td>1.2 ± 0.4</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>100 (\mu M) OXZ + 1.3 mM Ca(^{2+})</td>
<td>93.6 ± 2.7</td>
<td>790 ± 73</td>
</tr>
<tr>
<td>+ 1 mM CoCl(_2)</td>
<td>8.2 ± 3.7**</td>
<td>53 ± 4**</td>
</tr>
<tr>
<td>+ 1 mM NiCl(_2)</td>
<td>14.6 ± 0.6**</td>
<td>42 ± 1**</td>
</tr>
<tr>
<td>+ 1 mM MnCl(_2)</td>
<td>84.7 ± 4.0**</td>
<td>ND</td>
</tr>
<tr>
<td>+ 10 mM MnCl(_2)</td>
<td>6.5 ± 0.3**</td>
<td>ND</td>
</tr>
<tr>
<td>+ 1 mM quinine</td>
<td>44.6 ± 8.6**</td>
<td>164 ± 64**</td>
</tr>
</tbody>
</table>

Hemolysis and Ca\(^{2+}\) uptake were determined in separate experiments under the same experimental conditions. RBCs (2%) were incubated in the presence of 100 \(\mu M\) OXZ plus 1.3 mM Ca\(^{2+}\) with the additions indicated in the table. Hemolysis was determined at 30 min, and Ca\(^{2+}\) uptake was measured at 5 min in the presence of \(^{65}\)Ca\(^{2+}\) (1 \(\mu M\)/ml). Values are mean ± S.D. (n = 3—4), ND: not determined. *, **, Significantly different from the “100 \(\mu M\) OXZ + 1.3 mM Ca\(^{2+}\)” group at *; p<0.01 and **, p<0.001.

Ca\(^{2+}\) uptake increased linearly on increasing the medium Ca\(^{2+}\) concentration as measured at 1.5 and 5 min (Fig. 2B), indicating that Ca\(^{2+}\) is passively transported according to the concentration gradient. The degree of Ca\(^{2+}\) uptake paralleled that of hemolysis in terms of the OXZ and Ca\(^{2+}\) concentrations (Figs. 1B and C). Thus, hemolysis was preceded by Ca\(^{2+}\) influx.

Effects of Ca\(^{2+}\) Channel Blockers and Quinine As shown in Table 1, the non-selective Ca\(^{2+}\) channel blockers,\(^{16}\) Co\(^{2+}\) and Ni\(^{2+}\) (1 mM) and Mn\(^{2+}\) (10 mM), markedly inhibited hemolysis induced by 100 \(\mu M\) OXZ plus 1.3 mM Ca\(^{2+}\). Ca\(^{2+}\) uptake was also inhibited by the same concentration of Co\(^{2+}\) and Ni\(^{2+}\). Quinine (1 mM), an inhibitor of the Ca\(^{2+}\)-activated K\(^{+}\) channel,\(^{19}\) exhibited moderate inhibition of hemolysis and Ca\(^{2+}\) uptake (Table 1), whereas 1 mM quinidine, an isomer of quinine, did not inhibit hemolysis at all (data not shown).

Movement of Na\(^{+}\) and K\(^{+}\), and Effects of High-K\(^{+}\)/Low-Na\(^{+}\) Medium For analytical reasons, cellular Na\(^{+}\) and K\(^{+}\) concentrations were measured using 10% RBC suspension incubated with higher concentrations of OXZ in the presence and absence of Ca\(^{2+}\); both of these produced similar time–courses of hemolysis with about 75% hemolysis at 30 min (Figs. 3A and B). In the presence of Ca\(^{2+}\), OXZ reduced the cellular K\(^{+}\) content to about one third the control value and increased the Na\(^{+}\) content about 3-fold within 5 min, i.e., preceding the hemolysis (Fig. 3A). However, OXZ alone produced much smaller and gradual ionic changes (Fig. 3B).

On the other hand, under the regular conditions of 2% RBC, 1.3 mM Ca\(^{2+}\) and 100 \(\mu M\) OXZ, an increase in extracellular K\(^{+}\) concentration reduced the hemolysis and Ca\(^{2+}\) uptake, which was evident at the lower OXZ concentration of 80 \(\mu M\) (Table 2).

Morphological Observations by SEM As shown in Fig. 4, RBCs suspended in KH-Tris were largely composed of normal biconcave disk shaped cells, irrespective of the presence or absence of 1.3 mM Ca\(^{2+}\) ("Control"). Addition of 100 \(\mu M\) OXZ to the RBC suspension containing 1.3 mM Ca\(^{2+}\) ("100 \(\mu M\) OXZ + Ca\(^{2+}\)") caused rapid changes in morphology:
moderate invagination at 15 s, bumps and spicules in many cells at 30 s to 1 min, transformation to spherocynocytes and then formation of blebs with a reduction in size at 1—10 min, and pinching-off of blebs at 10—20 min. “100 μM OXZ” produced cubic and mouth-like invagination at 15 s which lasted up to 10 min and partially returned to the control morphology thereafter. With “400 μM OXZ,” deformation and reduction in size was already evident at 15 s and progressed thereafter without echinocytic changes.

Co²⁺ did not inhibit echinocyte formation but prevented bleb formation (“OXZ+Ca+Co”). Ni²⁺ was more effective, with some echinocytes being observed at 20 min (“OXZ+Ca+Ni”).

**SDS–PAGE Analysis of the Ghosts after Hemolysis**
In this experiment, we used human RBCs, which have been thoroughly characterized for cytoskeletal proteins.²⁰–²² In agreement with the reported differences in sensitivity of rat and human red cells to exogenous Ca²⁺, human RBCs required slightly higher OXZ concentrations to produce hemolysis than rat RBCs, i.e., 150 and 500 μM OXZ in the presence and absence of 1.3 mM Ca²⁺ respectively (data not shown). After incubation for 30 min, ghosts were prepared and subjected to SDS–PAGE (Fig. 5). Compared with the control RBCs incubated in the absence of OXZ (lanes 1 and 2) or in the presence of 150 μM OXZ alone (lane 3), the RBCs treated with OXZ/Ca²⁺ (lane 4) exhibited a non-electrophoresed band at the top of the separation gel and a marked reduction in spectrin bands, band 3 and 4.1 proteins, with the actin component being unchanged. This indicates that polymerization and liberation of membrane proteins had occurred. Hemolysis by 500 μM OXZ (lane 5) produced a broad staining through the band 3 to 4.2 proteins, but spectrin bands were almost unaltered.

**DISCUSSION**

OXZ was found to induce hemolysis in a Ca²⁺-dependent and -independent manner in vitro. Although amphipathic drugs generally cause hemolysis at high concentrations by their detergent-like action, enhancement of drug-induced hemolysis by Ca²⁺ is seldom reported; hemolysis induced by bile salts is enhanced at high Ca²⁺ concentrations, which is thought to be significant in the development of colon cancer.²⁴,²⁵ We also tested various drugs for their hemolytic action and Ca²⁺-dependence (data not shown). Of these drugs, prynylamine showed moderate Ca²⁺-dependence at 400 μM but not at 1 mM. Chlorpromazine and trifluoperazine, which cause hemolysis,¹⁰,¹² showed no Ca²⁺-dependence. Other

| Table 2. Effects of Extracellular Na⁺/K⁺ Concentrations on Hemolysis and Ca²⁺ Uptake Induced by OXZ |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Na⁺/K⁺ (mm) | 100 μM OXZ + 1.3 mm Ca²⁺ | 80 μM OXZ + 1.3 mm Ca²⁺ |
| Hemolysis (%) | Ca²⁺ uptake (nmol/ml RBCs) | Hemolysis (%) | Ca²⁺ uptake (nmol/ml RBCs) |
| 150/50 | 96.7±3.0 | 831±57 | 92.5±2.0 | 522±42 |
| 100/100 | 94.9±1.4 | 457±73 | 56.1±5.5** | 119±24** |
| 50/100 | 83.1±7.6* | 313±35** | 20.5±2.8** | 89±7** |
| 0/150 | 70.4±4.7** | 241±42** | 16.1±1.3** | 87±5** |

RBCs (2%) were suspended in a solution containing different concentrations of Na⁺/K⁺ and 20 mM Tris-HCl (pH 7.4). Hemolysis was determined 30 min after addition of OXZ plus 1.3 mm Ca²⁺. Ca²⁺ uptake was assayed at 5 min in the presence of ⁶⁰Ca²⁺ (1 μCi/ml). Values are means±S.D. (n=4), * p<0.01 and ** p<0.001.

**Fig. 4. Morphological Changes Induced by OXZ in the Presence and Absence of Ca²⁺, and Effects of Co²⁺ and Ni²⁺**
RBC suspensions (2%) were incubated with 100 μM OXZ plus 1.3 mm Ca²⁺, 100 μM OXZ without Ca²⁺, or 400 μM OXZ without Ca²⁺. Effects of 1 mm Co²⁺, Ni²⁺ and quinine were tested in the presence of 100 μM OXZ and 1.3 mm Ca²⁺. An aliquot was fixed at the times indicated. See the Materials and methods section for details. The bar indicates 10 μm.
tested drugs showing no hemolytic action at 0.1 and 1 mM, irrespective of the presence of Ca²⁺, were local anesthetics (procaine, lidocaine, dibucaine, tetracaine) and other membrane-acting drugs (procainamide, quinidine, propranolol, phenytoin, diphenhydramine, pyrillamine, mecridine, promethazine, imipramine, amitriptyline, verapamil, diltiazem, nicardipine, disopyramide). Of the anionic amphipathic drugs, ibuprofen, flurbiprofen and ethacrynic acid had no hemolytic action, whereas flufenamic acid induced Ca²⁺-dependent hemolysis at 1—2 mM. Thus, OXZ seems to be a rather novel drug that induces Ca²⁺-dependent hemolysis at comparatively low concentrations.

This Ca²⁺-dependent hemolysis may be initiated by the increased plasma membrane permeability for Ca²⁺, as shown by the marked increase in Ca²⁺ uptake preceding hemolysis. It is un conceivable that the radio-isotopic Ca²⁺ uptake is a result of an enhanced exchange reaction due to an increased intracellular free Ca²⁺ concentration which requires a release of bound Ca²⁺. The reason for this is that RBCs have no intracellular organ to pool calcium and, in addition, OXZ, because of its high lipid solubility, may dissolve in membrane lipids and may not be able to displace intracellular protein-bound calcium. Thus, we consider that Ca²⁺ uptake represents largely a net inward movement of Ca²⁺.

A rapid influx of Na⁺ and efflux of K⁺ occurred together with the Ca²⁺ influx. It is likely that influx of Na⁺ is critical for movement of Na⁺ and K⁺ and succeeding hemolysis, since the non-selective Ca²⁺ channel antagonists, Co²⁺ and Ni²⁺ inhibited the Ca²⁺ uptake by OXZ/Ca²⁺ concomitantly with suppression of hemolysis and morphological improvement. In addition, hemolysis was similarly induced by OXZ/Ca²⁺ in 0.15 M Tris–HCl buffer (pH 7.4) in the absence of Na⁺ and K⁺ (data not shown). However, Ca²⁺ influx by itself may be influenced by extracellular Na⁺ and K⁺, since enriched K⁺ medium inhibited both hemolysis and Ca²⁺ uptake induced by OXZ/Ca²⁺. This appears to agree with the partial inhibition of hemolysis and Ca²⁺ influx by quinine, an inhibitor of the Ca²⁺-activated K⁺ channel, which acts by displacing K⁺ from its external activation site and also inhibits Ca²⁺-induced vesiculation of RBC membranes.

The morphological changes induced by OXZ were quite different in the presence or absence of Ca²⁺. Fujii et al. reported that amphipathic drugs generally produce two types of morphological changes: drugs with a cationic group induce membrane internalization whereas those with an anionic group, like flufenamic acid, induce membrane externalization. This generalization applies to OXZ only in the absence of Ca²⁺. The successive morphological changes induced by OXZ/Ca²⁺, i.e., 1) transformation to echinocytes, 2) membrane blebbing, and 3) pinching-off of the blebs, may be initiated by influx of Ca²⁺ and the ensuing movement of Na⁺ and K⁺, and proceed with the irreversible derangement of the cytoskeletal components of plasma membranes as shown by the SDS–PAGE patterns of the damaged ghosts, e.g., appearance of polymerized proteins and reduction in spectrin bands. Bleb formation is an established phenomenon in Ca²⁺-dependent hepatocyte death. However, the echinocytic transformation by itself may not be sufficient to induce hemolysis, since Co²⁺ and Ni²⁺ also were unable to prevent echinocytic transformation despite the fact that they markedly inhibited Ca²⁺ uptake and hemolysis. These observations are similar to those reported with the Ca²⁺-ionophore, A23187, which is known to induce echinocyte formation accompanied by RBC rigidity and loss of deformability. It also causes Ca²⁺-mediated cytoskeletal damage involving cross-linking of membrane proteins, protocytosis, and tyrosine phosphorylation of band 3 protein.

Hemolysis by OXZ alone involves different mechanisms, since 1) it required a higher OXZ concentration and was not inhibited by 1 mM Co²⁺ and Ni²⁺ or 10 mM Mn²⁺ (data not shown), 2) movement of Na⁺ and K⁺ during the course of hemolysis was much less and slower compared with that of OXZ/Ca²⁺, and in addition, 3) morphological changes were quite different from those in the presence of Ca²⁺. Clearly, Ca²⁺ has a critical role in exacerbating hemolysis.

The mechanisms for the increased Ca²⁺ permeability induced by OXZ remain to be determined. OXZ not only has a unique chemical structure, i.e., 2 molecules of an adrenergic agent (mephenytine) combined with a cholinergic agent (ethanolamine) via an amide linkage, but this molecule also contains highly hydrophobic side-chains at both ends and hydrophobic groups in the center (Fig. 6). Thus, we presume that OXZ molecules would dissolve into the membrane lipid layer by hydrophobic binding and form hydrophilic pores or paths, like a Ca²⁺ ionophore. This Ca²⁺ ionophore-like action may require higher concentrations of OXZ than that for local anesthetic action, since hemolysis occurred only above 60 μM irrespective of RBC concenentrations (Fig. 1A), whereas the local anesthetic action of OXZ is manifested at less than 10 μM. At higher toxic doses, however, the unphysiological or toxicological significance of the Ca²⁺-mediated actions of OXZ will not be neglected. Finally, the increase in portal pressure by OXZ in the isolated rat liver was inhibited by co-infusion of 1 mM of non-selective Ca²⁺-channel metal antagonists, the inhibition being 91.1±2.4, 66.8±5.5 and 46.3±4.7 (% mean±S.D., n=3), for Co²⁺, Mn²⁺ and Ni²⁺, respectively. OXZ, therefore, possibly accelerates Ca²⁺ entry into smooth muscle cells of the portal vein, but this remains to be proven.
In conclusion, OXZ is a rare drug with a Ca\(^{2+}\) ionophore-like action, and it increases the membrane Ca\(^{2+}\) permeability of RBCs and possibly of other cells to cause toxic or unphysiological Ca\(^{2+}\)-mediated reactions.

Acknowledgements The authors thank Dr. Hajime Katabayama, Department of Pharmaceutical Chemistry, NCP for his helpful discussions on the steric configuration of OXZ, and they also thank Dr. Hiroshi Uraakami, Department of Microbiology, NCP, for his help with scanning electron microscopy techniques.

REFERENCES