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Lidocaine action and conformational changes in cytoskeletal protein network in human red blood cells

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Abstract

The mechanism of action of lidocaine, which is commonly used clinically as a local anesthetic, was studied in human red blood cells. The influx of [^{14}C]lidocaine through the cell membrane induced reversible transformation of human red blood cells from discocytes to stomatocytes. This change in shape depended on the lidocaine concentration and required both ATP and carbonic anhydrase. The lidocaine-induced shape change occurred as a result of spectrin aggregation, which altered the intracellular environment of the human red blood cells, mediated by carbonic anhydrase and activation of vacuolar type H^+-ATPase (V-ATPase). Lidocaine controlled the influx of ^{22}Na into the human red blood cells in a concentration-dependent manner. When incubated in media containing 6-chloro-9-[(4-diethylamino)-1-methyl-butyl]amino-2-methoxyacridine (mepacrine), an inhibitor of Na^+ channels, human red blood cells changed shape from discocytes to stomatocytes and the intracellular pH decreased. This phenomenon was very similar to the shape change induced by lidocaine. These results suggest that the mode of action of lidocaine is related to a conformational change in the cytoskeletal protein network.

Keywords: Lidocaine; Spectrin; ATPase, vascular type; Carbonic anhydrase; Erythrocyte membrane, human

1. Introduction

Local anesthetics are known to block Na^+ channels and stabilize cell membranes in a similar manner in nerves (Narahashi et al., 1969; Hille, 1977; Courtney, 1978), skeletal muscles (Schwartz et al., 1977) and cardiac tissues (Bean et al., 1983; Clarkson et al., 1988). The mechanism of Na^+ channel blockage has been explored previously with respect to both membrane lipids (Hille, 1977; Yeagle et al., 1977; Ehring et al., 1988) and membrane proteins (Chan and Wang, 1984; Matsumoto et al., 1984; Angelides et al., 1988; Srinivasan et al., 1988). It is generally accepted that anesthetics permeate through the membrane in uncharged form and undergo reprotonation on reaching the cytoplasm to exert their effects. These drugs inhibit sodium permeation by directly binding to the Na^+ channels on the cytoplasmic side of the excitable membrane (Narahashi et al., 1970; Narahashi and Frazier, 1971). The blocking mechanism, however, remains unclarified in relation to the behavior of Na^+ channels. The cytoskeletal protein network has been reported to play a role as a regulatory factor in structural changes in Na^+ channels in squid giant axons (Matsumoto et al., 1984) and in neurons of the rat brain (Srinivasan et al., 1988; Angelides et al., 1988). For further studies, the erythrocyte membrane is useful as a model system, since it has a basic structure similar to that of the general cell, and the structure of the cytoskeletal network of the cytosol resembles that in axons.

Therefore, we used human red blood cells to study the mechanism of action of the local anesthetic lidocaine. A change in human red blood cell shape from discocytes to stomatocytes was seen after the application of local anesthetic. The mechanism of this change was studied in relation to the cytoskeletal network.

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2. Materials and methods

2.1. Materials

Chemicals: 3-[*N*-morpholino]propanesulfonic acid (MOPS), *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (Hepes), nigericin and 6-chloro-9-[(4-diethylamino)-1-methyl-butyl]amino-2-methoxyacridine (mepacrine) were obtained from Sigma Chem. Co., St. Louis, MO, USA. The following chemicals were purchased from the sources indicated: lidocaine hydrochloride (Fujisawa Pharm. Co., Kanagawa, Japan); [^{14}C]lidocaine hydrochloride, [^{32}P] and [^{22}Na] (New England Nuclear, Boston, MA, USA); 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 3'-acetyl-2'-carboxyethyl-6',7'-(dihydropyran-2'-one)-5-carboxyfluorescein diacethoxymethylester (BCECF-AM; Dojindo Laboratory, Kumamoto, Japan). Other reagents were obtained from Wako Pure Chem. Ind. Osaka, Japan).

2.2. Preparation of human red blood cell and human red blood cell membranes

Venous blood was drawn from a healthy adult volunteer, immediately heparinized and then centrifuged at $2000 \times g$ for 20 min to remove plasma and the buffy coat. human red blood cells in the pellet were washed three times with phosphate-buffered saline (10 mM phosphate buffer, 145 mM NaCl, pH 7.4; PBS) and then suspended in PBS. Washed human red blood cells were hemolysed in 50 volumes of ice-cold 10 mM Tris-HCl (pH 7.4) and then centrifuged at $15\,000 \times g$ in a Kubota centrifuge (KR-2000, RA-3) at 0°C for 30 min. The membrane fraction was obtained by repeating the same procedure with ice-cold 10 mM Tris HCl (pH 7.4).

2.3. Isolation of spectrin

Spectrin was isolated from the human red blood cell membrane preparation as described previously (Morrow et al., 1980). The membrane extract (15–18 mg of protein) obtained after incubation with 10 volumes of 0.1 mM EDTA (pH 8.0) at 4°C for 5 h was subjected to Sepharose CL-4B column chromatography (2.5×90 cm), and eluted with 10 mM Tris-HCl, 0.5 mM 2-mercaptoethanol, and 0.2 mM diisopropylfluorophosphate at a flow rate of 0.6 ml/min.

2.4. Observation of changes in shape

The changes in shape of human red blood cells were observed by scanning electron microscopy (JEOL, JSM-35). Specimens were prepared as described previously (Nishiguchi et al., 1993). human red blood cells were fixed with 1% glutaraldehyde in PBS, followed by routine alcohol dehydration.

2.5. Influx of [^{14}C]lidocaine into human red blood cells

Washed human red blood cells were incubated in PBS containing 10.5 mM lidocaine and 1.85 MBq/ml [^{14}C]lidocaine for 30 s, 5 min, 15 min, or 30 min at 37°C (Ht: 10%), and centrifuged at $2000 \times g$ for 5 min at 4°C. The pellets were washed twice with PBS and hemolysed in 10 volumes of ice-cold 10 mM Tris-HCl (pH 7.4). The hemolysate solution were centrifuged at $15\,000 \times g$ for 30 min at 4°C. The radioactivity in the supernatants was measured using a scintillation counter (Aloka, LSC-903).

2.6. Influx of ^{22}Na into human red blood cells

Washed human red blood cells were incubated with PBS containing 0 mM, 3.1 mM, 10.5 mM or 21.8 mM lidocaine and 370 KBq/ml ^{22}Na for 5 min, 15 min or 30 min at 37°C (Ht: 10%), and centrifuged at $2000 \times g$ for 5 min at 4°C. The pellets were washed twice with PBS and hemolysed in 10 volumes of ice-cold 10 mM Tris-HCl (pH 7.4). The hemolysate solutions were centrifuged at $15\,000 \times g$ for 30 min at 4°C. The radioactivity in the supernatants was measured using a scintillation counter.

2.7. Electrophoresis

Protein was electrophoresed as reported by Laemmli (1970). Samples were analyzed on 3–10% linear gradient polyacrylamide gels under nonreducing conditions. Gels were stained with Coomassie brilliant blue R-250, and the intensities of the stained bands were measured using a Personal Densitometer PD110 (Molecular Dynamics Co., Tokyo, Japan).

2.8. Preparation of antibodies against human erythrocyte spectrin dimers

Two rabbits were immunized with three injections each of mixtures of purified spectrin from human red blood cells and Freund's adjuvant. The first injection was given subcutaneously with a mixture of 1 mg of purified spectrin in 500 μl of PBS and Freund's complete adjuvant (ratio 1:1 v/v). After 4 weeks, a mixture of 1 mg of purified spectrin in 500 μl of PBS and Freund's incomplete adjuvant (ratio 1:1 v/v) was injected intramuscularly. After a further 2 weeks, the final injection was given in the same manner as the second, and the rabbits were bled two weeks later. Affinity-purified anti-spectrin antibodies were prepared on a column of purified spectrin-coupled Affigel-10 (Bio Rad) (Shimizu et al., 1990).

2.9. Immunofluorescence analysis

Smears of human red blood cells were fixed briefly in acetone, washed in PBS and then incubated with a 1:20 dilution of primary antiserum in a humid chamber at 37°C for 30 min. After being rinsed in PBS, the specimens were incubated in FITC-labeled goat antiserum raised against rabbit IgG. A fluorescence microscope was used for immunohistochemical observation.

2.10. Measurement of intracellular pH (pHi) in human red blood cells using BCECF-AM

pHi was determined using the intracellularly trappable fluorescent pH indicator BCECF-AM (Musgrove et al., 1986; Bright et al., 1987). Washed human red blood cells, at a cell density of 8% hematocrit, were labeled with 10 μM BCECF-AM in Hepes buffer (153 mM NaCl, 5 mM KCl, 5 mM glucose, 20 mM Hepes, pH 7.4) for 60 min at 37°C. After incubation, the cells were washed three times with PBS at $2000 \times g$ for 5 min each time at 4°C. The fluorescence of BCECF was measured using a FACScan (Becton-Dickinson, Mountain View, CA) flow cytometer at excitation and emission wavelengths of 495 nm and 530 nm, respectively.

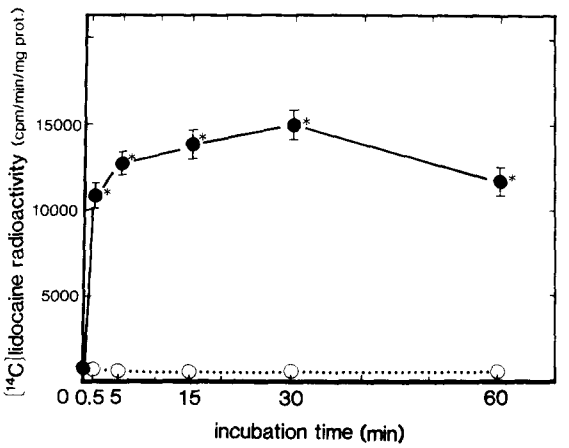

Fig. 1. Influx of [^{14}C]lidocaine into human red blood cells. Human red blood cells were incubated with PBS containing 10.5 mM lidocaine and 1.85 MBq/ml [^{14}C]lidocaine for 30 s, 5 min, 15 min, 30 min or 60 min at 37°C (●), and washed 3 times with PBS after incubation in media containing 10.5 mM lidocaine and 1.85 MBq/ml for 15 min at 37°C (○) (Ht: 10%). Values are means ± S.D. for three experiments. Significantly different from control $^{*}P < 0.001$.

The pH was calculated by comparing the ratio determined in the sample to a standard curve constructed using cells exposed to high-KCl buffer (130 mM KCl,

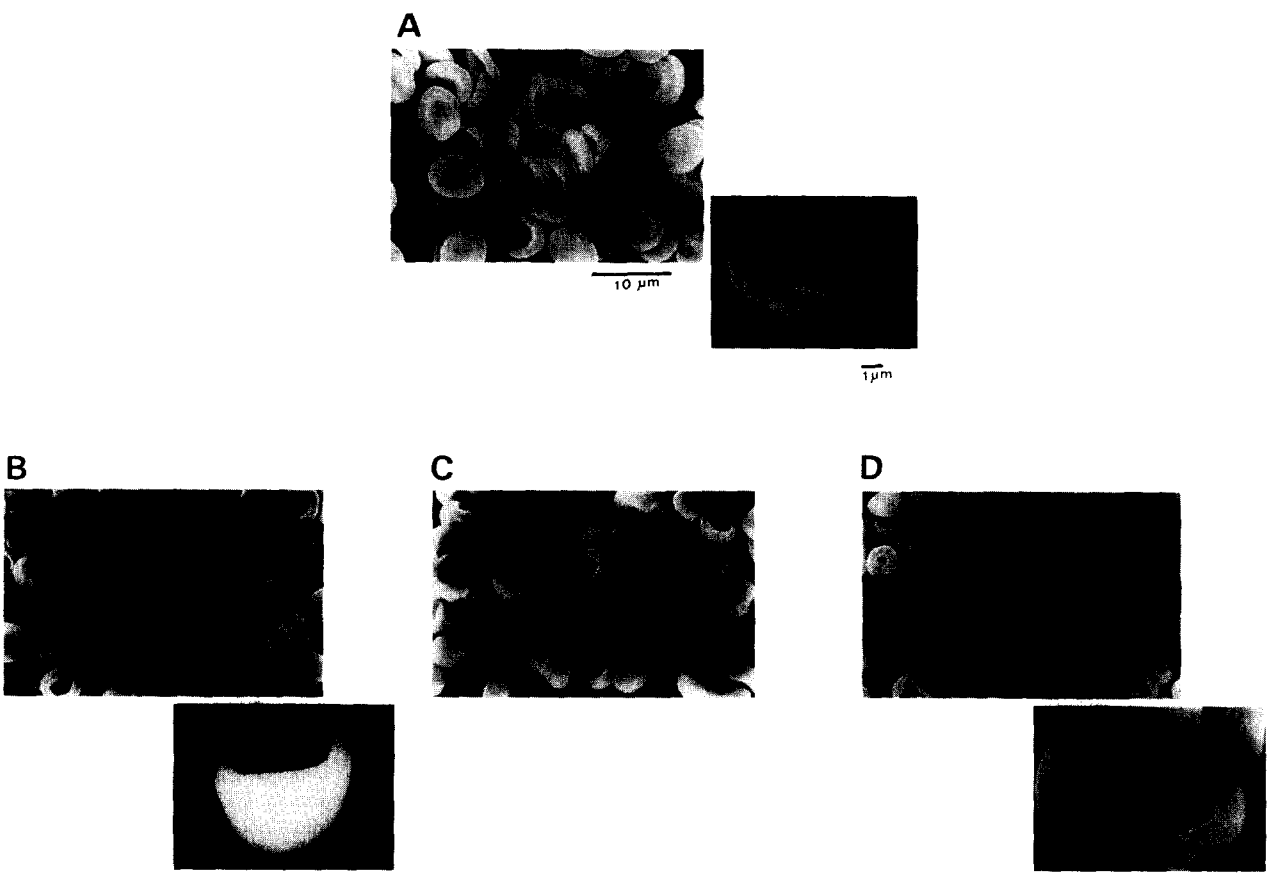

Fig. 2. Scanning electron micrographs of human red blood cells incubated in media containing lidocaine at concentrations of 0 mM (A), 3.3 mM (B), 10.5 mM (C) and 20.1 mM (D) at 37°C for 15 min (Ht: 10%).

10 mM NaCl, 1 mM $MgSO_4$, 10 mM Na-MOPS) at various pHs (pH 6.0, 6.4, 6.7, 7.0, 7.4) in the presence of 10 μg/ml of the ionophore nigericin, which causes equilibration of pHi with that of the buffer, for 15 min (Thomas et al., 1979). The mean fluorescence was determined by computer analysis of the obtained histograms.

3. Results

3.1. Influx of [^{14}C]lidocaine into human red blood cells and lidocaine-induced human red blood cell shape change

As shown in Fig. 1, high levels of [^{14}C]lidocaine were counted in the cells immediately after its addition, and the amount incorporated reached saturation at 30 min. Lidocaine was removed from the cytoplasm by washing the cells three times with PBS.

Human red blood cells were incubated in media containing 0 mM, 3.3 mM, 10.5 mM, or 20.1 mM lidocaine for 15 min at 37°C, and their shape changed from discocytes to stomatocytes. This shape change was dependent on the concentration of lidocaine (Fig. 2).

3.2. Effects of lidocaine on the influx of ^{22}Na into human red blood cells

Influx of ^{22}Na into human red blood cells incubated with PBS containing 0 mM, 3.1 mM, 10.5 mM or 21.8 mM lidocaine and 370 kBq/ml ^{22}Na for 5 min, 15 min or 30 min at 37°C decreased with increasing concentrations of lidocaine (Fig. 3).

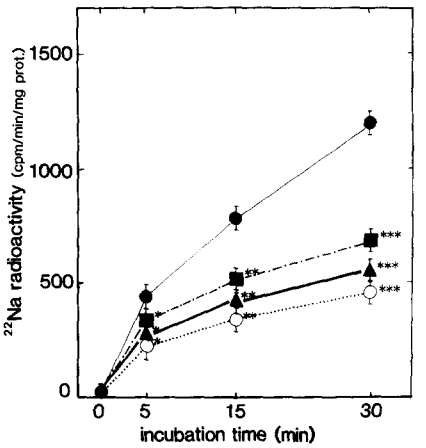

Fig. 3. Influx of ^{22}Na into human red blood cells. Human red blood cells were incubated in media containing 0 mM (●), 3.1 mM (■), 10.5 mM (▲) or 21.8 mM (○) lidocaine and 370 kBq/ml ^{22}Na for 5 min, 15 min or 30 min at 37°C (Ht: 10%). Values are means ± S.D. for three experiments. Significantly different from control $^{*}P < 0.05$, $^{**}P < 0.02$ and $^{***}P < 0.01$.

3.3. Measurement of pHi in human red blood cells with lidocaine

BCECF-AM-labeled washed human red blood cells were incubated with PBS containing 0 mM, 1 mM, 10 mM, 15 mM or 20 mM lidocaine at 25°C for 5 min, 15 min, 30 min or 60 min, and the pHi was determined by flow cytometry. The pHi of human red blood cells decreased as the concentration of lidocaine increased at each incubation time (Fig. 4).

3.4. Effects of mepacrine on human red blood cells

When incubated in PBS containing 100 μM mepacrine, a Na^+ channel inhibitor, for 5 min at 37°C, the shape of human red blood cells changed from discocytes to stomatocytes (Fig. 5A). The pHi decreased on incubation with PBS containing 0 μM, 100 μM, 200 μM or 300 μM mepacrine for 5 min, 15 min, 30 min or 60 min in a concentration- and time-dependent manner (Fig. 5B).

3.5. Effects of bafilomycin A_1, an inhibitor of H^+-ATPase, on the lidocaine-induced shape change and pHi

To determine the effects of inhibitors of the vacuolar-type H^+-ATPase (V-ATPase) on the lidocaine-induced shape change in human red blood cells, bafilomycin A_1, a specific inhibitor of V-ATPase, was used. When human red blood cells were incubated in buffer containing 0 mM or 21.0 mM lidocaine or 10 μM bafilomycin A_1 for 30 min at 37°C, they showed no

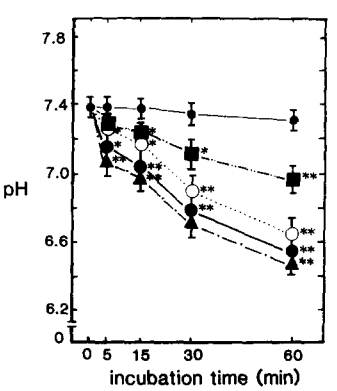

Fig. 4. Changes in pHi of lidocaine-treated human red blood cells. BCECF-loaded human red blood cells were incubated in media containing 0 mM (●), 1 mM (■), 10 mM (○), 15 mM (•) or 20 mM (▲) lidocaine for 5 min, 15 min, 30 min or 60 min at 37°C (Ht < 1%). Values are means ± S.D. for six experiments. Significantly different from control $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

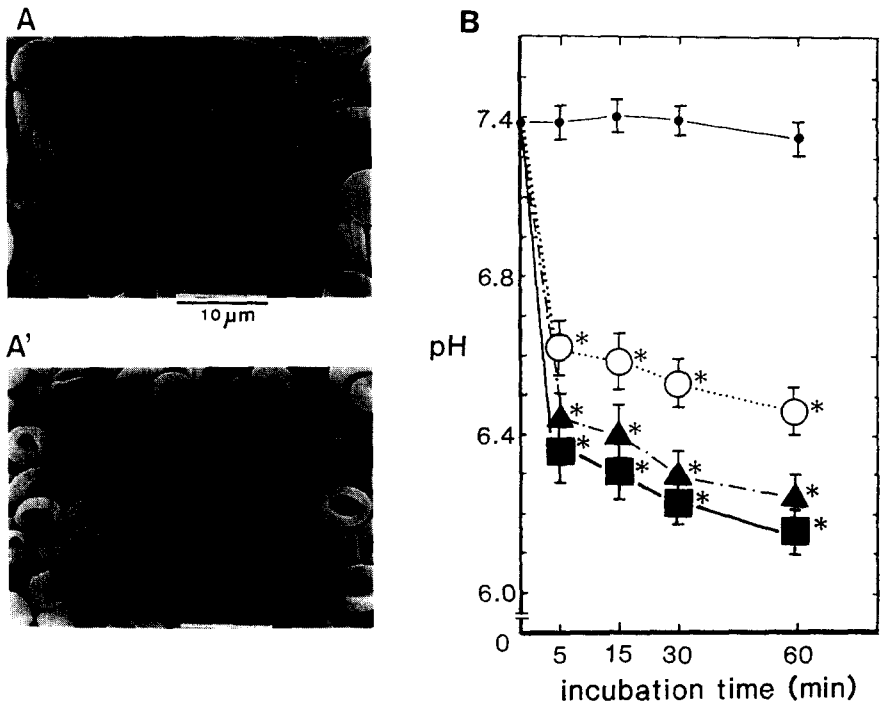

Fig. 5. Effects of mepacrine on the shape and pHi of human red blood cells. Scanning electron micrographs of human red blood cells incubated in media containing 0 μM (A) or 100 μM mepacrine (A') for 5 min at 37°C (Ht: 10%). These scanning electron micrographs are representative of three experiments using different human red blood cell preparations with identical results. (B) BCECF-loaded human red blood cells were incubated in media containing 0 μM (●), 100 μM (○), 200 μM (▲) or 300 μM (■) mepacrine for 5 min, 15 min, 30 min or 60 min at 37°C (Ht < 1%). Values are means ± S.D. for three experiments. Significantly different from control $^{*}P < 0.001$.

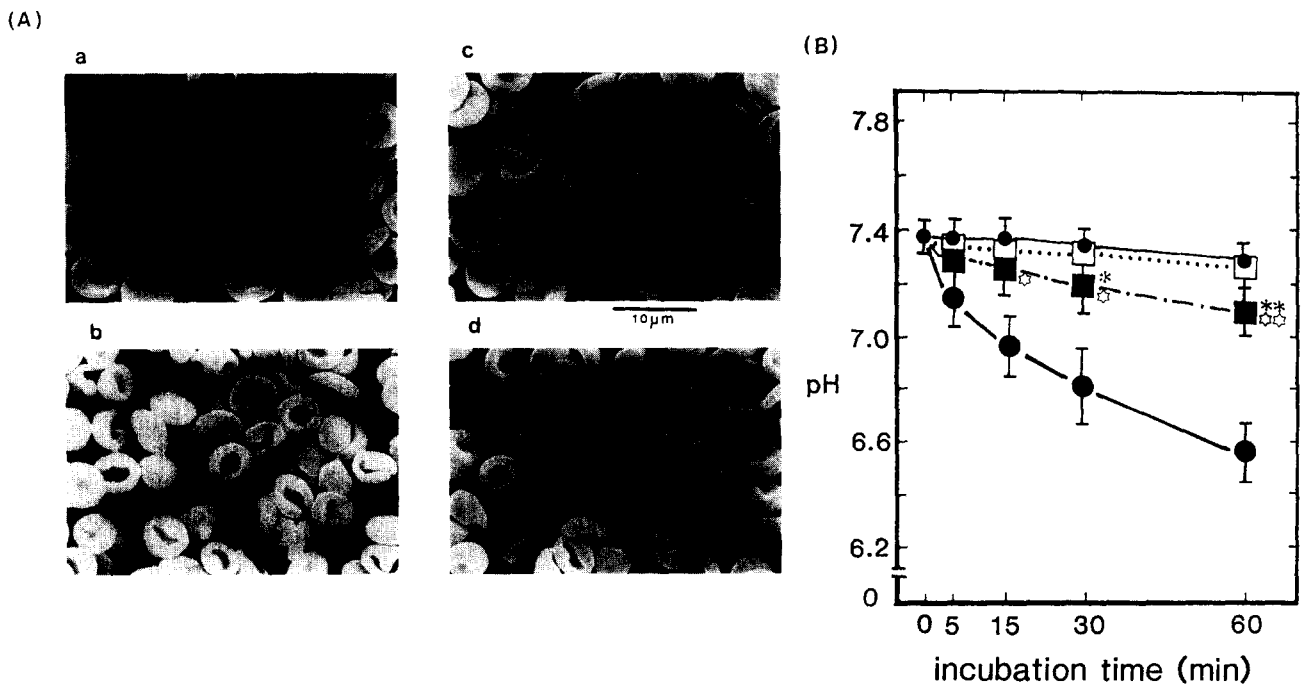

Fig. 6. Effects of bafilomycin A_1 on the shape and pHi of lidocaine-treated human red blood cells. (A) Human red blood cells were incubated in media containing 0 mM (a) or 21.0 mM lidocaine (b) for 30 min at 37°C, with 10 μM bafilomycin A_1(c) for 10 min at 37°C followed by incubation with 21.0 mM lidocaine for 30 min at 37°C (d) (Ht: 10%). (B) Changes in pHi of lidocaine-treated erythrocytes. Human red blood cells were incubated in media containing 0 mM (●), 14.3 mM lidocaine (●), 1 μM bafilomycin A_1 (□) or 1 μM bafilomycin A_1 plus 14.3 mM lidocaine (■) (Ht < 1%). Values are means ± S.D. for six experiments. Significantly different from control $^{*}P < 0.05$ and $^{**}P < 0.02$. Significantly different from lidocaine-treated group $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$.

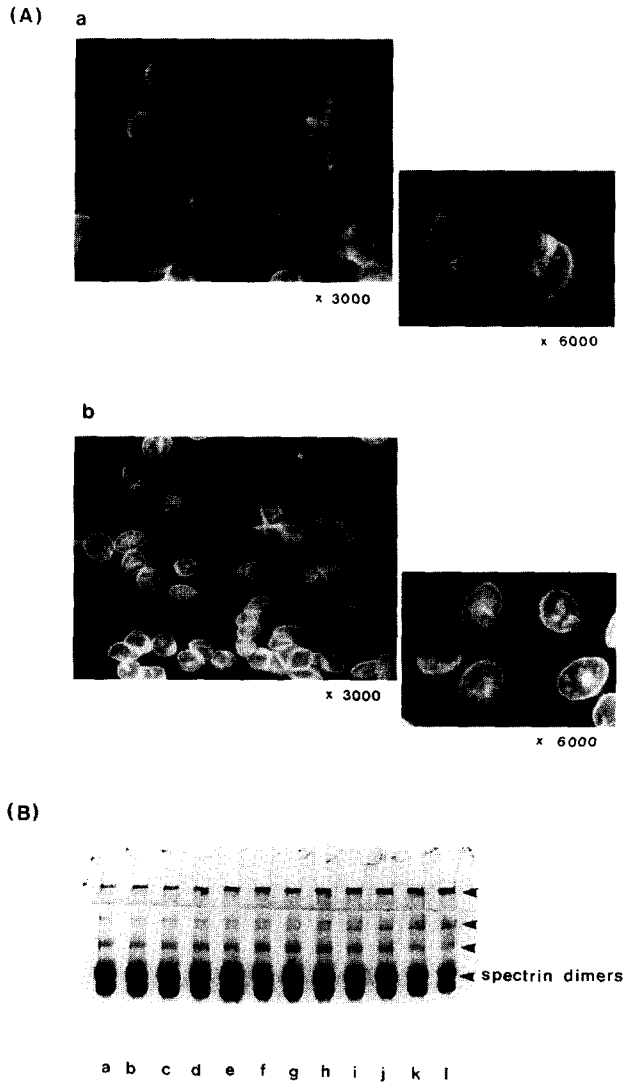

Fig. 7. (A) Lidocaine-induced changes in spectrin distribution in human red blood cells. Indirect immunofluororescence micrographs of human red blood cells incubated at 37°C for 5 min in: (a) PBS; (b) PBS containing 10.1 mM lidocaine (Ht: 10%). (B) Analysis by SDS-PAGE of spectrin dimers incubated at 37°C for 30 min in PBS at pH 7.4 (a), 7.0 (b), 6.9 (c), 6.8 (d), 6.7 (e), 6.6 (f), 6.5 (g), 6.4 (h), 6.3 (i), 6.2 (j), 6.0 (k) or 5.5 (l). The pH of the buffer was adjusted with 0.1 M HCl. Samples were analyzed on 3–10% linear gradient gels under non-reducing conditions.

change in shape. Incubation with 21.0 mM lidocaine for 30 min at 37°C after preincubation in buffer containing 10 μM bafilomycin A_1 for 10 min at 37°C induced a slight shape change compared with untreated human red blood cells (Fig. 6A). When human red blood cells were incubated in buffer containing 1 μM bafilomycin A_1 for 10 min at 37°C, pHi did not change in comparison with that of controls. However, on incubation with 14.3 mM lidocaine for 5 min, 15 min, 30 min or 60 min at 37°C after preincubation in buffer containing 1 μM bafilomycin A_1 for 10 min at 37°C, the human red blood cell intracellular pHi showed a slight decrease compared with that in untreated cells (Fig. 6B).

3.6. Spectrin aggregation in lidocaine-treated human red blood cells and effects of pH on extracted spectrin dimers

To determine whether the lidocaine-induced shape change was related directly to spectrin, its distribution in lidocaine-treated human red blood cells was observed by indirect immunofluorescence with anti-spectrin antibodies. Aggregation of fluorescence was observed in lidocaine-treated cells (Fig. 7Ab), while the fluorescence was distributed diffusely in untreated (Fig. 7Aa) and in cells treated with both acetazolamide and lidocaine (data not shown). Diffuse fluorescence was also observed in cells washed after treatment with lidocaine (data not shown).

Isolated spectrin dimers were incubated with PBS at various pHs at 37°C for 20 min and analyzed by SDS-PAGE. The bands of spectrin dimers and tetramers decreased, while that of the high molecular weight spectrin species increased with decreasing pH of the buffer (Fig. 7B).

4. Discussion

The mechanism of action of lidocaine was investigated in relation to conformational changes in the cytoskeletal protein network in human red blood cells. The lidocaine-induced shape change of human red blood cell occurred dose and time dependently. Lidocaine was incorporated into human red blood cells dependent on the concentration of lidocaine (Nishiguchi et al., 1989). Furthermore, [^{14}C]lidocaine was removed from the cytoplasm by washing. Lidocaine has been reported to block the Na^+ channel (Narahashi et al., 1970; Cahalan and Almers, 1979). The effect of sodium on the lidocaine-induced shape change was investigated. The influx of Na^+ into human red blood cells was observed to be controlled by the lidocaine concentration. We used a high concentration of lidocaine because the membrane potential of human red blood cells is low. Influx of ^{22}Na into human red blood cells decreased with increasing concentrations of lidocaine. When human red blood cells were incubated in media containing mepacrine, an inhibitor of Na^+ channels (Cantiello et al., 1990), they changed shape from discocytes to stomatocytes. This phenomenon was very similar to the shape change induced by lidocaine. The flow of ions as a result of conformational changes in ion channels was observed as an influx of ^{32}P into the human red blood cells. The influx of ^{32}P into the

human red blood cells was controlled in a manner dependent on the concentration of lidocaine and was very similar to that observed with addition of DIDS, a specific inhibitor of anion channels (Cabantchik et al., 1978) (data not shown). Therefore, we consider that the observed lidocaine-induced shape change was related to the flow of ions. The lidocaine-induced shape change appeared 30 s after lidocaine application and was dependent on both the concentration of lidocaine and the incubation period. The shape change required ATP levels higher than approximately 0.4 mM and carbonic anhydrase in the cytoplasm, and was reversible. Furthermore, this shape change was considered to be related to spectrin aggregation (Nishiguchi et al., 1993). The same results were observed when human red blood cells were incubated with other local anesthetics (data not shown). We considered the shape change to be a manifestation of the action of lidocaine, and examined the structural changes in spectrin and the possible roles of carbonic anhydrase and ATP accompanying this effect of lidocaine.

Carbonic anhydrase catalyzes the reversible hydration of carbon dioxide ($CO_2 + H_2O \rightleftarrows HCO_3 + H^+$) (Lindskog and Coleman, 1973), hydrolysis of certain esters, and a variety of other reactions (Verpoorte et al., 1967). As lidocaine, a basic tertiary amine, is only marginally soluble in water, it is usually prepared as its water-soluble salt, lidocaine hydrochloride, in which it exists as both uncharged molecules and as positively charged substituted ammonium cations in aqueous solution. Local anesthetics, on passing through the cell membrane in an uncharged form, are assumed to become cationic in the immediate vicinity of nerve-membrane receptors (Narahashi et al., 1970). It has been reported that local anesthetics are more effective in blocking action potentials at low as compared to high pH (Catchlove, 1972). It is likely that they block the action potentials as protonated molecules inside the membrane. Therefore, we investigated the pHi of lidocaine-treated and untreated human red blood cells using BCECF-AM and flow cytometry; lidocaine reduced the pHi of human red blood cells in a dose-dependent manner. In contrast, human red blood cells preincubated with acetazolamide, an inhibitor of carbonic anhydrase (Pocker and Stone, 1968), prior to incubation with various concentrations of lidocaine showed no such change in pH. Furthermore, mepacrine reduced the pHi of human red blood cells in a same manner.

We investigated the role of ATP in the human red blood cell shape change induced by lidocaine in relation to H^+-ATPase (V-ATPase) function. Erythrocytes incubated with lidocaine after incubation in media containing bafilomycin A_1, a specific inhibitor of V-ATPase (Bowman et al., 1988; Yoshimori et al., 1991), showed a slight change in shape and a small reduction in pHi compared with cells treated only with lidocaine. A V-ATPase has been reported to regulate cytoplasmic pH in murine macrophages (Swallow et al., 1990). The results of the present study suggest that human red blood cells possess V-ATPase which is activated by lidocaine, inducing H^+ influx and, as a consequence, the observed changes in pHi.

The inner side of the human red blood cell membrane is laminated by a protein network composed of spectrin, actin, band 4-1 and other proteins (Branton et al., 1981; Cohen, 1983). This multiprotein network combines with ankyrin, which is itself associated with band 3 and seems to maintain the mechanical stability of the plasma membrane and to control cell shape and deformability (Beaven and Gratzer, 1980). Spectrin, which is composed two nonidentical subunits, is the major component of the human red blood cell membrane skeleton, and spectrin heterodimers associate noncovalently head to head to form tetramers and oligomers (Liu et al., 1987). It has been reported that spectrin dimers can self-associate into tetramers (Ungewickell and Gratzer, 1978) and oligomers (Morrow and Marchesi, 1981), and that the spectrin dimer-tetramer equilibrium depends on the ionic strength, temperature, and spectrin concentration (Ungewickell and Gratzer, 1978; Liu and Palek, 1980). When the distribution of spectrin in intact cells was analyzed by indirect immunofluorescence, aggregation of fluorescence was observed in lidocaine-treated cells, possibly indicating spectrin aggregation. In contrast, diffuse immunofluorescence was observed in untreated, acetazolamide-treated and PBS-washed lidocaine-treated cells (data not shown), presumably due to the normal ball-shaped multi-protein network (Liu et al., 1987). Spectrin dimers isolated from human red blood cells and incubated with PBS at various pHs followed by SDS-PAGE analysis showed an increase in the proportion of spectrin oligomers with decreasing pH. From these results, we consider that spectrin aggregation is induced by the acidic intracellular environment in human red blood cells as a result of increased H^+ concentration in response to lidocaine, presumably mediated by carbonic anhydrase and activation of V-ATPase.

In conclusion, we propose that the mode of action of lidocaine is related to conformational changes in the cytoskeletal protein network as a result of spectrin aggregation.

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