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Regulation of erythrocyte ghost membrane mechanical stability by chlorpromazine

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Abstract

Chlorpromazine (CPZ), a widely used tranquilizer, is known to induce stomatocytic shape changes in human erythrocytes. However, the effect of CPZ on membrane mechanical properties of erythrocyte membranes has not been documented. In the present study we show that CPZ induces a dose-dependent increase in mechanical stability of erythrocyte ghost membrane. Furthermore, we document that spectrin specifically binds to CPZ intercalated into inside-out vesicles depleted of all peripheral proteins. These findings imply that CPZ-induced mechanical stabilization of the erythrocyte ghost membranes may be mediated by direct binding of spectrin to the bilayer. Membrane active drugs that partition into lipid bilayer can thus induce cytoskeletal protein interactions with the membrane and modulate membrane material properties. © 2001 Published by Elsevier Science B.V.

Keywords: Chlorpromazine; Erythrocyte; Membrane stability; Spectrin

1. Introduction

Chlorpromazine (CPZ) is widely used as a tranquilizer in the management of certain psychiatric disorders. The amphipathic feature of CPZ enables it to rapidly distribute itself into the lipid bilayer of biological membranes and in the case of human erythrocytes induces morphologic transformation from a normal biconcave disc to a stomatocytic form [1–6]. During passage through the microvasculature, the circulating erythrocytes undergo extensive passive deformation and marked alterations in cell shape. This entails the erythrocyte membrane to undergo large extensional deformation without membrane fragmentation. The unique mechanical features of human erythrocytes, a readily deformable yet a mechanically stable membrane, enable the erythrocyte to undergo the required deformations without losing structural integrity. The unique mechanical properties of the erythrocyte membrane are primarily regulated by the spectrin-based membrane skeleton that underlies the lipid bilayer [7]. Marked extensibility of the spectrin lattice accounts for membrane deformability while lateral interactions of spectrin with actin, protein 4.1R and adducin in the skeletal network are responsible for maintaining membrane mechanical stability. The spectrin-based network is anchored to the bilayer through interactions with two transmembrane proteins, band 3 and glycophorin C [8,9], and

Abbreviations: CPZ, chlorpromazine; IOV, inside-out vesicle; DI, deformability index; DI_{max}, maximal value of DI

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these protein–protein interactions have recently been shown to play a role in maintaining membrane mechanical stability [10,11]. Spectrin can also directly interact with phosphatidylserine in the lipid bilayer [12,13], but the role of this protein–lipid interaction in regulating membrane mechanical properties has not been elucidated. Furthermore, no information is currently available on the potential interaction between bilayer intercalating drugs such as CPZ and various membrane skeletal proteins and the effects of such interactions on membrane mechanical properties. In the present study, we examined whether CPZ can affect the mechanical properties of erythrocyte ghost membranes. We show that CPZ induces a dose-dependent increase in membrane mechanical stability. We further document that spectrin specifically binds to CPZ intercalated into inside-out vesicles depleted of all peripheral proteins. These findings imply that CPZ mechanically stabilizes erythrocyte membranes by inducing binding of spectrin to the bilayer. We have thus identified a novel role for membrane active drugs that partition into lipid bilayer in mediating cytoskeletal protein interactions with the membrane and thereby modulating membrane mechanical function.

2. Experimental procedures

2.1. Materials

A 10 mM stock solution of chlorpromazine hydrochloride (Wako Pure Chemical Industries) was prepared in a medium containing 150 mM KCl, 1 mM $MgCl_2$ and 1 mM dithiothreitol adjusted to pH 6.9 using Tris and stored at 4°C in the dark.

2.2. Preparation of erythrocyte ghosts

After obtaining informed consent, venous blood was obtained from healthy volunteer donors. Erythrocytes isolated from whole blood were washed three times with Tris-buffered wash solution (10 mM Tris, 120 mM KCl, pH 7.4). Intact erythrocytes were lysed and washed three times in 35 vols. of hypotonic buffer (5 mM Tris, 5 mM KCl, pH 7.4) at 4°C. The resulting white ghost membranes were gently stirred at 0°C for 10 min in buffer A (150 mM KCl, 1 mM $MgCl_2$ and 1 mM dithiothreitol, pH 7.4) and subsequently incubated at 37°C for 40 min to allow membrane resealing [14]. Resealed ghosts were incubated with various concentrations of CPZ at 0°C for 10 min. The morphology of glutaraldehyde fixed resealed ghosts was examined by dark-field light microscopy using a Nikon microscope. At a CPZ concentration of 1.5 mM, approx. 100 nM of CPZ was incorporated into 1 mg of ghost membranes.

2.3. Measurement of membrane mechanical properties

Resealed ghost preparations were resuspended in a 35% dextran solution and membrane deformability was measured using an ektacytometer, as previously described [14,15]. Briefly, ghosts were subjected to increasing levels of applied shear stress (0–150 dynes/cm^2) and changes in the deformability index (DI), a direct measure of cell ellipticity, was recorded as a function of applied stress. The maximal value of DI attained (DI_{max}) is a measure of the surface area of resealed ghosts while the initial slope of the DI curve provides a measure of membrane deformability. To determine membrane mechanical stability, resealed ghosts suspended in a 50% dextran solution were subjected to a constant applied shear stress of 750 dynes/cm^2 in the ektacytometer. The rate of decrease of DI, a direct measure of the rate of membrane fragmentation, provides a quantitative measure of membrane mechanical stability [16].

2.4. Measurement of membrane associated CPZ

To quantitate the amount of CPZ associated with resealed membranes, ghosts incubated with 1.5 mM CPZ were diluted with various volumes of buffer A. The membrane suspension was centrifuged at $7500 \times g$ for 10 min and the amount of CPZ in the supernatants was quantified by HPLC using a 5C18-MS Packed Column (Cosmosil). The amount of ghost membrane associated CPZ was calculated by subtracting the amount of CPZ in the supernatant from the total amount of added CPZ.

2.5. Measurement of spectrin binding to CPZ-loaded inside-out vesicles

Inside-out vesicles (IOV) depleted of all peripheral

proteins including spectrin were prepared as described by Danilov et al. [17]. Spectrin dimers were purified from human erythrocytes as described by Harris et al. [18]. IOVs at a protein concentration of 0.83 mg/ml were incubated with various concentrations of CPZ at 0°C for 10 min. Thereafter, samples containing 100 μg of IOVs were incubated with varying concentrations of spectrin dimers in 250 μl of buffer B (130 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.4) at 24°C for 1 h. Following incubation, 200 μl of the mixture was layered on top of 200 μl of 8% sucrose solution in buffer B in a centrifuge tube. Following centrifugation at $35\,000\times g$ at 4°C for 40 min, the pelleted IOVs were collected and their protein content analyzed electrophoretically using 7.5% sodium dodecyl sulfate–polyacrylamide gels. Coomassie brilliant blue stained gels were scanned using a densitometer (Fluor-S Multiimager, Bio-Rad) and the extent of spectrin associated with IOVs quantitated.

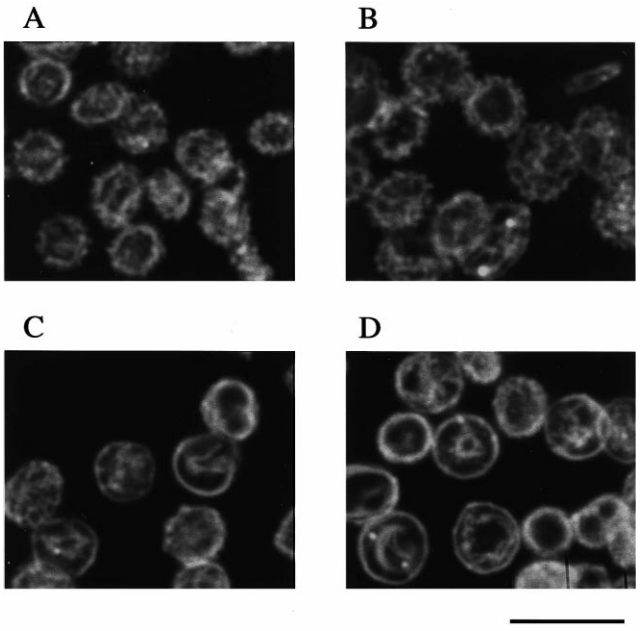

Fig. 1. Effects of CPZ on the shape of resealed ghosts. The morphology of resealed ghosts incubated with 0 (A), 0.6 (B), 1.4 (C) and 2.1 (D) mM CPZ was observed as described in Section 2. The bar indicates 10 μm.

3. Results

3.1. Effect of CPZ on ghost morphology and membrane deformability

To determine whether CPZ induces stomatocytic shape changes in ghosts as it does in intact erythrocytes, we examined the morphology of CPZ-treated ghosts. While control ghosts without CPZ were echinocytic, the ghosts treated with increasing concentrations of CPZ at 0°C for 10 min became progressively more stomatocytic (Fig. 1), implying that CPZ induces similar morphological changes in ghosts as has been previously reported for intact erythrocytes [19,20]. Higher concentrations of CPZ were needed to induce stomatocytic changes in ghosts compared to discoid erythrocytes since an additional amount of CPZ was needed to convert the initially echinocytic ghosts into discoid shape before transformation into stomatocytes. The effect of CPZ on membrane deformability was assessed by ektacytometry (Fig. 2). The deformability profiles of ghost membranes incubated with CPZ at concentrations up to 1.5 mM were very similar to that of control membranes regardless of the extent of induced stomatocytic shape changes. These findings imply that CPZ at concentrations up to 1.5 mM does not alter membrane deformability. However, treatment with higher CPZ concentrations resulted in decreased DI_{max}, indicating irreversible loss of functional surface area. This is most likely the result of the generation of spherostomatocytes with endovesicles. Similar effects of CPZ on loss of cellular deformability of intact erythrocytes due to endocytosis at high concentrations have previously been reported [21].

3.2. Effects of CPZ on ghost membrane mechanical stability

Representative data from membrane mechanical stability measurements on ghosts treated with various concentrations of CPZ are shown in Fig. 3. When the control ghosts were exposed to a shear stress of 750 dynes/cm^2, they rapidly reached their maximum extent of deformation (a DI value of 0.82). However, with continued application of this high level of shear stress, the ghosts began to fragment resulting in progressive decrease of DI. The rate of decline in DI is a direct measure of the rate of membrane fragmentation. The rate of decline of

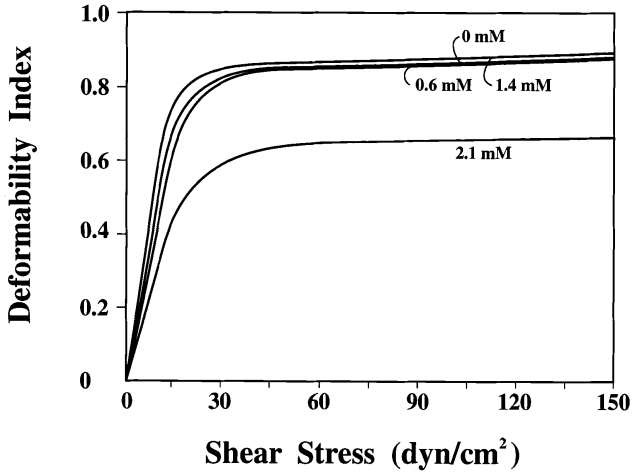

Fig. 2. Effects of CPZ on membrane deformability. Membrane deformability of resealed ghosts incubated with various concentrations of CPZ was measured using an ektacytometer as described in Section 2.

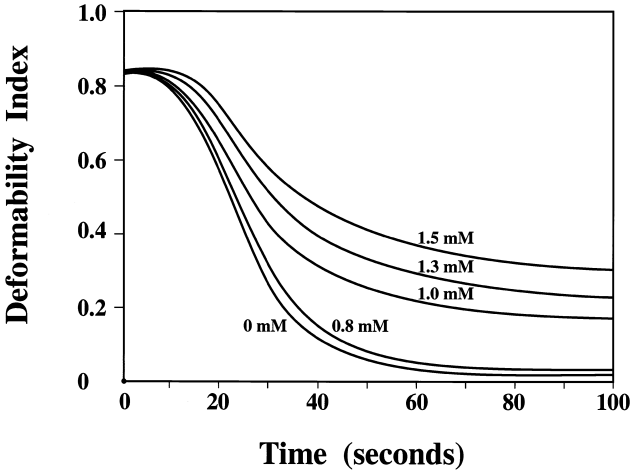

Fig. 3. Effects of CPZ on membrane mechanical stability. Membrane stability of resealed ghosts incubated with various concentrations of CPZ was measured using an ektacytometer as described in Section 2.

DI for ghost membranes treated with CPZ at concentrations <0.8 mM was very similar to that of control ghosts, indicating that CPZ at these concentrations has little effect on ghost membrane mechanical stability. However, at higher CPZ concentrations there was a dose-dependent decrease in the rate of DI decline, implying that CPZ at concentrations between 0.8 and 1.5 mM increases membrane mechanical stability.

3.3. Reversible effects of CPZ on membrane mechanical stability

To confirm that the observed increase in membrane mechanical stability is indeed due to CPZ intercalating into the membrane, we explored the reversibility of the induced change following extraction of CPZ from the membrane. Ghosts incubated with 1.5 mM CPZ were diluted with various volumes of buffer A and the residual amount of CPZ associated with the ghosts was quantitated. Representative data for the distribution of CPZ between the supernatant and the pellet in each sample following different dilutions are shown in Fig. 4A. With increasing dilution there is progressive decrease in the amount of membrane associated CPZ. Following a 10-fold dilution, the amount of CPZ associated with membranes decreased by 55%. In parallel, membrane mechanical stability decreased reaching a level comparable to that of untreated ghosts (Fig. 4B). These findings imply that CPZ-induced changes in mechanical stability are completely reversible.

3.4. Effects of CPZ on spectrin binding to IOVs

To investigate whether CPZ can mediate spectrin binding to biological membranes, we quantitated binding of purified spectrin to IOVs incubated with various concentrations of CPZ (Fig. 5A). Control IOVs and IOVs preloaded with CPZ at 0.8 mM or

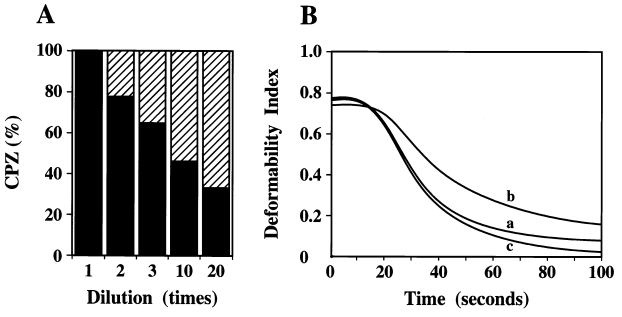

Fig. 4. Extraction of CPZ from erythrocyte ghosts. (A) Ghosts incubated with 1.5 mM CPZ were diluted with various volumes of buffer A. The amount of CPZ distributed in the ghost membranes was determined as described in Section 2. (B) Membrane stability of samples shown in A were measured using an ektacytometer (a, control ghosts without CPZ; b, ghosts incubated with 1.5 mM CPZ; c, 1.5 mM CPZ-loaded ghosts following a 10-fold dilution with buffer A).

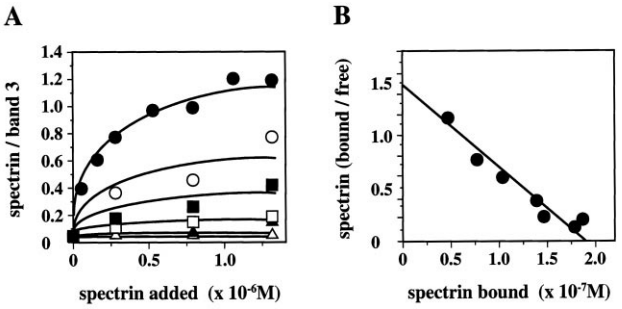

Fig. 5. Effects of CPZ on spectrin binding to IOV. (A) Spectrin at various concentrations was incubated with IOV preloaded with 0 (△), 0.4 (▲), 0.8 (□), 1.0 (■), 1.5 (○), and 1.6 (●) mM CPZ. The amount of spectrin bound to IOV was determined as described in Section 2. (B) Scatchard analysis of the data derived from incubation with 1.6 mM CPZ (A, ●). The estimated K_d value and binding capacity were 130 nM and 239 μg/mg, respectively.

less bound very little spectrin and the extent of binding did not increase with increasing concentrations of added spectrin. Importantly, preloading of IOVs with CPZ at concentrations greater than 0.8 mM and up to 1.6 mM resulted in a marked increase in the extent of spectrin binding. Spectin binding to these IOVs was concentration dependent and saturable. Scatchard analysis of spectrin binding to IOVs preloaded with 1.6 mM CPZ (Fig. 5B) showed that spectrin binds to CPZ with a moderately high affinity (K_d = 130 nM). Our calculations suggest that spectrin interaction with the membrane occurs when 1000 CPZ molecules per spectrin dimer are loaded onto the IOV membrane. In contrast to spectrin, CPZ had no effect on protein 4.1 or ankyrin binding to IOVs (data not shown).

4. Discussion

The present study identified a novel effect of CPZ on the mechanical properties of erythrocyte ghost membrane. Over a range of concentrations, CPZ induced a dose-dependent increase in membrane mechanical stability with little effect on membrane deformability. Importantly, the CPZ effect on ghost membrane stability was completely reversible. Previous studies [8,9] have shown that mechanical stability of erythrocyte membranes is regulated by protein–protein interactions, lateral interactions of spectrin with other skeletal proteins, and by the vertical interaction of spectrin with band 3 mediated by ankyrin. We have now been able to document that spectrin association with lipid bilayer mediated by CPZ can also regulate membrane mechanical stability. These findings thus enabled the identification of an important role for spectrin–lipid interaction in modulating membrane function.

In the present study we obtained direct evidence for spectrin interaction with CPZ intercalated into the bilayer. Detailed analysis of the binding data revealed that spectrin binds to CPZ with a moderately high affinity – K_d on the order of 100 nM. It is interesting to note that the binding affinity of spectrin to CPZ is very similar to the binding affinity of spectrin to ankyrin and to protein 4.1R. Previous studies have shown that spectrin can bind to negatively charged phospholipid, phosphatidylserine localized to the inner leaflet of the bilayer. The CPZ-induced change in membrane stability we documented is unlikely to be the consequence of CPZ-induced association of spectrin with phosphatidylserine. This is because phosphatidylserine is scrambled in the bilayer of ghosts prepared in the absence of MgATP [22–24] and we have been able to document that spectrin cannot bind to any significant extent to IOVs in which phosphatidylserine was scrambled. It should also be noted that CPZ itself has potential to scramble lipid distribution in the bilayer [6,25,26]. More importantly, we have been able to document that removal of CPZ from ghost membranes reversed the induced effects on membrane mechanical stability. Thus the observed effects can be attributed directly to interaction of the skeletal network with CPZ in the lipid bilayer.

Human erythrocyte membranes constitute a useful model to study the mechanism of drug action on membranes. Spectrin analogues have been identified in a variety of non-erythroid cells including various cell types in the brain [27,28]. Although the tranquilizing effect of CPZ is most likely the result of blocking the monoamine receptors of nerve cells [29,30], the CPZ-induced association of spectrin-based membrane skeleton with the plasma membrane of the neuronal cells may represent an additional mechanism of its action.

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References

[1] B. Deuticke, Biochim. Biophys. Acta 163 (1968) 494–500.
[2] M.P. Sheetz, S.J. Singer, Proc. Natl. Acad. Sci. USA 71 (1974) 4457–4461.
[3] T. Fujii, T. Sato, A. Tamura, M. Wakatsuki, Y. Kanaho, Biochem. Pharmacol. 28 (1979) 613–620.
[4] B. Isomaa, H. Hagerstrand, G. Paatero, Biochim. Biophys. Acta 899 (1987) 93–103.
[5] H. Hagerstrand, M. Danieluk, M.B. Hagerstrand, A. Iglic, A. Wrobel, B. Isomaa, M. Nikinmaa, Biochim. Biophys. Acta 1466 (2000) 125–138.
[6] J.Y. Chen, W.H. Huestis, Biochim. Biophys. Acta 1323 (1997) 299–309.
[7] N. Mohandas, J.A. Chasis, Semin. Hematol. 30 (1993) 171–192.
[8] V. Bennett, Physiol. Rev. 70 (1990) 1029–1065.
[9] D.M. Gilligan, V. Bennett, Semin. Hematol. 30 (1993) 74–83.
[10] P.S. Low, B.M. Willardson, N. Mohandas, M. Rossi, S.B. Shohet, Blood 77 (1991) 1581–1586.
[11] X.-L. An, Y. Takakuwa, W. Nunomura, S. Manno, N. Mohandas, J. Biol. Chem. 271 (1996) 33187–33191.
[12] C.W.M. Haest, G. Plasa, D. Kamp, B. Deuticke, Biochim. Biophys. Acta 509 (1978) 21–32.
[13] C. Mombers, P.W.M. Van Dijck, L.L.M. Van Deenen, J. De Gier, A.J. Verkleij, Biochim. Biophys. Acta 470 (1977) 152–160.
[14] B.P. Heath, N. Mohandas, J.L. Wyatt, S.B. Shohet, Biochim. Biophys. Acta 691 (1982) 211–219.
[15] J.A. Chasis, N. Mohandas, J. Cell Biol. 103 (1986) 343–350.
[16] N. Mohandas, M.R. Clark, B.P. Heath, M. Rossi, L.C. Wolfe, S.E. Lux, S.B. Shohet, Blood 59 (1982) 768–774.
[17] Y.N. Danilov, R. Fennell, E. Ling, C.M. Cohen, J. Biol. Chem. 265 (1990) 2556–2562.
[18] H.W. Harris Jr., S.E. Lux, J. Biol. Chem. 255 (1980) 11512–11520.
[19] N. Mohandas, C. Feo, Blood Cells 1 (1975) 375–384.
[20] E. Nishiguchi, J. Sindo, N. Hamasaki, Biochim. Biophys. Acta 1176 (1993) 95–105.
[21] M. Bessis, N. Mohandas, Blood Cells 1 (1975) 315–321.
[22] R.F.A. Zwaal, A.J. Schroit, Blood 89 (1997) 1121–1132.
[23] S.L. Schrier, A. Zachowski, P. Herve, J.-C. Kader, P.F. Devaux, Biochim. Biophys. Acta 1105 (1992) 170–176.
[24] B. Verhoven, R.A. Schlegel, P. Williamson, Biochim. Biophys. Acta 1104 (1992) 15–23.
[25] C. Moreau, J.C. Sulpice, P.F. Devaux, A. Zachowski, Mol. Membr. Biol. 14 (1997) 5–12.
[26] H. Hagerstrand, T.H. Holmstrom, M. Bobrowska-Hagerstrand, J.E. Eriksson, B. Isomaa, Mol. Membr. Biol. 15 (1998) 89–95.
[27] V. Bennett, J. Davis, W.E. Fowler, Nature 299 (1982) 126–131.
[28] R.K. Sihag, J. Neurochem. 71 (1998) 2220–2228.
[29] J.M. Van Rossum, Arch. Int. Pharmacodyn. 160 (1966) 492–494.
[30] P. Seeman, Pharmacol. Rev. 32 (1981) 229–313.