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Original Article

Prehemolytic Impact of Phenothiazine Drugs on the Attachment of Spectrin Network in Red Blood Cells

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Abstract

Introduction: Chlorpromazine, thioridazine, and trifluoperazine are phenothiazine drugs that cause colloid-osmotic hemolysis of human erythrocytes by unknown mechanism. To clarify this mechanism, the impact of these drugs on the β_{sp} (1.4 MHz) and γ_{1sp} (9 MHz) dielectric relaxations was investigated. Each relaxation was shown to reduce its strength on the severing specific bridge that connects the spectrin network with the lipid membrane. For β_{sp} relaxation, this is the spectrin-actin-glycophorin C bridge while for γ_{1sp} relaxation this is the spectrin-ankyrin-band 3 bridge.

Aim: To elucidate the mechanisms of the effects of phenothiazine drugs in prehemolytic concentrations on the red blood cell plasma membrane using scanning temperature-dependent (thermal) differential dielectric spectroscopy.

Materials and methods: Erythrocytes were isolated from freshly drawn blood and 100 μl of them were suspended in 1 ml isotonic solution of 10 mM NaCl and mannitol (working medium) containing the indicated concentration of the drug for 10 min at 23°C. The treated erythrocytes were isolated, suspended in working medium, hematocrit 0.55, and heated (heating rate 1.5°C/min) above the denaturation temperature of spectrin (T_A≈49.5°C) in order to obtain the differential dielectric spectroscopy data. The complex admittance, $Y^* = Y' + j.Y''$, of the tested suspensions was continuously measured and separated into its real (Y') and imaginary (Y'') parts using Solartron 1260 Impedance Frequency Analyzer.

Results: At pre-hemolytic concentrations, each drug inhibited these two relaxations, predominantly the γ_{1sp} relaxation. The results could be interpreted in terms of a sigmoid effect of the drugs on the spectrin-ankyrin-band 3 bridge severing it at concentration just prior to the start of massive hemolysis.

Conclusions: The study points at the possible mechanism of erythrocyte damage after treatment with phenothiazine drugs at prehemolytic concentrations. This is probably due to the disruption of the bridges between the phospholipid bilayer and the submembrane spectrin network.

Keywords

dielectric relaxations, drug-induced hemolysis, erythrocyte membrane

INTRODUCTION

The plasma membrane of human erythrocytes consists of a network of mainly spectrin tetramers that supports the lipid membrane and contains two major integral proteins (band 3 and glycophorin C).[1] The spectrin network (membrane skeleton, MS) is attached to the lipid membrane by the so-called spectrin-ankyrin-band 3 bridge and by the spectrin-actin-glycophorin C bridge.

Chlorpromazine (CPZ), thioridazine (TRZ) and trifluoperazine (TFP) are tricyclic phenothiazine drugs used in the treatment of mental anxiety and disorders.[2] These drugs exhibit a two-phase action on erythrocyte membranes. At low concentrations, they stabilize the membranes and cause stomatocytosis of erythrocytes[3,4], while at high concentrations, they induce colloid-osmotic hemolysis[5] by an unknown mechanism. To study this mechanism, we investigated the impact of CPZ, TRZ, and TFP on recently detected β_{sp} and γ_{1sp} dielectric relaxations on the spectrin network of erythrocytes.[6,7] A recent report[6] has shown that β_{sp} relaxation is inhibited by specific severing of spectrin-actin-glycophorin C bridge while $\gamma1_{sp}$ relaxation was inhibited by specific severing of spectrin-ankyrin-band 3 bridge. Based on this, the presented results could be interpreted by a sigmoid effect of studied drugs on the spectrin-ankyrin-band 3 bridge severing it just prior to the onset of hemolysis.

AIM

The aim of this study was to elucidate the mechanisms of the effects of phenothiazine drugs in pre-hemolytic concentrations on the red blood cell plasma membrane using thermal dielectric spectroscopy.

MATERIALS AND METHODS

Materials

NaCl, mannitol, chlorpromazine, thioridazine, and trifluoperazine were purchased from Sigma Chemicals Co, St. Louis, MO, USA.

Treatment of erythrocytes with phenothiazine drugs

Erythrocytes were isolated from freshly drawn blood as described previously.[6,7] 100 μl of the erythrocytes were suspended in 1 ml isotonic solution of 10 mM NaCl and mannitol (working medium) containing the indicated concentration of the drug for 10 minutes at room temperature.[3] After the treatment, the level of hemolysis was evaluated spectrophotometrically at 600 nm. Prior to heating, the cells were isolated and re-suspended in the working medium, hematocrit 0.55.

Scanning temperature-dependent differential dielectric spectroscopy of erythrocyte suspensions at the spectrin denaturation temperature, T_A

The obtained suspensions were immediately heated (from 30°C to 60°C with a heating rate of 1.5°C/min) across the T_A (49.5°C) in order to obtain the differential dielectric spectroscopy at 16 frequencies between 20 kHz and 15 MHz with an integration time of 0.5 s data associated with the denaturation of spectrin as previously described.[6,7] The complex admittance, $Y^* = Y' + j.Y''$, of tested suspension was continuously measured and separated into its real (Y') and imaginary (Y'') parts using Solartron 1260 Impedance Frequency Analyzer.

The complex admittance, Y^*, of heated suspension has been shown to change sharply at T_A and the obtained changes strongly depended on frequency (f).[6,7] These changes $\Delta Y'(f)$ and $\Delta Y''(f)$, were corrected for the linear effect of temperature and were defined as the value at the native minus the value at the denatured state of spectrin. They were ascribed to the spectrin's admittance contribution that was eliminated at T_A. The complex plain plot, $\Delta Y''$ vs. $\Delta Y'$, depicted two perfect semicircle arcs **(Fig. 1A)**, one placed above and another one below the real axis.[6,7] The upper arc expressed the β_{sp} relaxation associated with a piezo effect powered by the electrostriction of the lipid membrane. The lower arc revealed the $\gamma1_{sp}$ relaxation associated with the resonance of electric field with the natural oscillations of dipoles (segments) of spectrin network.

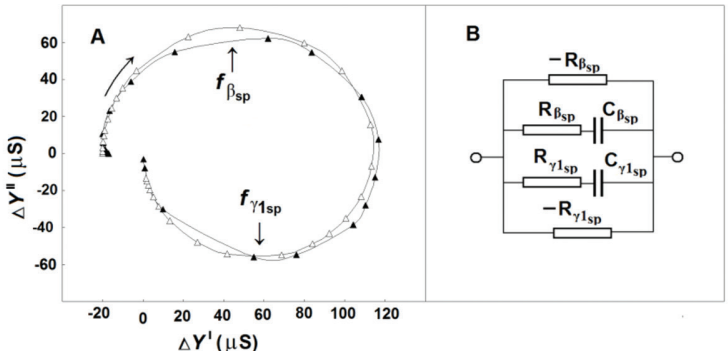

Figure 1. Complex plain plot (▲) of the admittance contribution of spectrin network of suspended erythrocytes (**A**). Curved arrow indicates the increase in frequency from 20 kHz to 15 MHz. Arrows indicate the characteristic frequencies of β_{sp} and $\gamma1_{sp}$ dielectric relaxations. Open triangles (Δ) indicate the model plot, Y" vs. Y', of the complex admittance, Y*, of equivalent electric circuit (**B**).

Model representation of β_{sp} and γ_{1sp} relaxations in erythrocytes

To obtain quantitative description of relaxations, we used an adequate electric model **(Fig. 1B)**. The $R_{\beta sp}$ and $C_{\beta sp}$ of upper circuit represented the best-fit values for the β_{sp} relaxation, while the $R_{\gamma 1sp}$ and $C_{\gamma 1sp}$ of lower circuit were the best-fit values for the γ_{1sp} relaxation. The complex admittance, $Y^* = Y'+jY''$, of the model circuit was obtained by iteration and the model plot, Y" vs. Y', depicted two semicircles which pertained to the admittance change plot, $\Delta Y''$ vs. $\Delta Y'$, of spectrin network as previously reported.[7] The $R_{\beta sp}$, $C_{\beta sp}$, $R_{\gamma 1sp}$, and $C_{\gamma 1sp}$ parameters linearly depended on hematocrit when its values varied between 0.10 and 0.60. Hence, it was convenient to use the ratios of these parameters. Such are the energy dissipation ratio, $-R_{\gamma 1sp}/R_{\beta sp}$ and the energy storage ratio, $-C_{\gamma 1sp}/C_{\beta sp}$, which represent the amount of energy dissipated and stored, respectively, on spectrin during the γ_{1sp} relaxation relative to that in β_{sp} relaxation. Compared to control erythrocytes, the $-R_{\gamma 1sp}/R_{\beta sp}$ ratio increased, while the $-C_{\gamma 1sp}/C_{\beta sp}$ ratio decreased strongly in case the spectrin-band 3 attachment bridge was severed.[6,7]

RESULTS

Fig. 2 shows the effects produced by various concentrations of TFP on the complex plot, $\Delta Y''$ vs. $\Delta Y'$, of spectrin's admittance contribution for erythrocytes pre-treated with TFP. The TFP treatment induced either non- or insignificant hemolysis. Nevertheless, the $\Delta Y''$ vs. $\Delta Y'$ plot demonstrated that TFP inhibited both relaxations whereat the γ_{1sp} relaxation was much stronger reduced compared to β_{sp} relaxation. For each concentration of TFP, the model plot was also obtained (not shown) and the best-fit values of its parameters are given in **Table 1**.

The data in **Table 1** indicate that at concentrations up to 200 μM TFP weakly and linearly changed the model parameters, $R_{\beta sp}$ and $C_{\beta sp}$, for the β_{sp} relaxation. By contrast, for the γ_{1sp} relaxation these parameters demonstrated sigmoid variation being almost constant up to 150 μM and sharply changing at 200 μM **(Fig. 2, right)**. These results suggest that TFP-treatment of erythrocytes induced predominant detachment of spectrin-ankyrin-band 3 bridge. At the same time, the TFP-induced hemolysis was zero at concentrations below 150 μM, weak at 200 μM, and complete at 250 μM (data not shown).

The erythrocytes treated with TRZ and CPZ produced similar results to those reported above for TFP (not shown). Compared to TFP, the latter drugs differed by the concentrations at which they induced hemolysis and specifically inhibited the γ_{1sp} relaxation. Up to the concentrations, where hemolysis became noticeable, the parameters of β_{sp} relaxation changed weakly and linearly while those of γ_{1sp} relaxation sustained marked sigmoid changes suggesting disruption of band 3-ankyrin-spectrin attachment bridge followed by massive hemolysis. The prehemolytic concentration interval was 200-250 μM for TFP, 400-450 μM for TRZ, and 1000-1200 μM for CPZ in line with the previous report.[3]

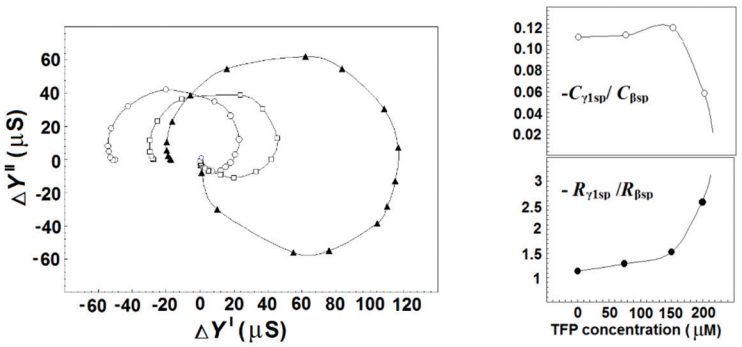

Figure 2. Left: complex plain plot of spectrin's admittance contribution for erythrocytes treated by TFP at concentrations 0 (▲), 150 μM (□), and 200 μM (○). **Right**: Effect of TFP concentration on the $-R_{\gamma 1sp}/R_{\beta sp}$ and $-C_{\gamma 1sp}/C\beta_{sp}$ ratios of TFP-treated erythrocytes.

Table 1. Model study of the effect produced by TFP on the β_{sp} and γ_{1sp} relaxations in erythrocytes. Only the mean values are shown, the deviations were less than 10% of the mean values. Both relaxations are expressed by their characteristic frequencies, $f_{\beta sp}$ and $f_{\gamma 1sp}$, and the best-fit values of their RC circuits **(Fig. 2B)**

Concentration of TFP (μM)	$R_{\beta sp}$ (kOhm)	$C_{\beta sp}$ (pF)	$f_{\beta sp}$ (MHz)	$R_{\gamma 1sp}$ (kOhm)	$C_{\gamma 1sp}$ (pF)	$f_{\gamma 1sp}$ (MHz)	$-R_{\gamma 1sp}/R_{\beta sp}$	$-C_{\gamma 1sp}/C_{\beta sp}$
0	−5.9	−23	1.2	6.7	2.5	8.0	1.13	0.11
75	−7.1	−19.4	1.2	9.2	2.2	7.5	1.3	0.11
150	−10.1	−14	1.1	15.4	1.7	6.5	1.52	0.12
200	−10.5	−13	1.2	27.1	0.74	8.0	2.57	0.06

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DISCUSSION

The causes for destabilization of the erythrocyte membrane by phenothiazine drugs could be multiple. However, one of them could be elucidated taking into account the accompanying changes in the $-R_{\gamma 1sp}/R_{\beta sp}$ and $-C_{\gamma 1sp}/C_{\beta sp}$ ratios. According to a previous study[5], these changes suggest that spectrin-ankyrin-band 3 bridge was specifically severed just prior to the onset of massive hemolysis. This outcome could be attributed to the predominant incorporation of positively charged phenothiazines into the inner, negatively charged, leaflet of the membrane bilayer[3,4], thereby disturbing the balance of spectrin-lipid membrane interactions. The latter conclusion is supported by the report[8] that the dissociation of spectrin-ankyrin-band 3 bridge leads to the decomposition of spectrin tetramers into constituent dimers impairing the mechanical function of spectrin network.

There are another two findings in line with the above conclusion. Electron microscopy of CPZ-treated erythrocytes has indicated almost two times greater width of their plasma membranes compared to intact erythrocytes.[5] This could be due to the increased number of spectrin dimers as each dimer is about 100 nm long and could be connected to the membrane only at one of its two ends. Freeze-fracture electron microscopy of the membrane of CPZ-treated erythrocytes revealed multiple groups of aggregated intramembrane particles (integral proteins) into the surrounding particle-free patches of bilayer membrane.[5] In general, such intramembrane particle aggregation is regarded as a consequence of the structural disturbance of MS.

CONCLUSION

This study suggests a possible mechanism responsible for the disturbance of MS and the membrane of erythrocytes by their treatment with prehemolytic concentrations of phenothiazine drugs.

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Прегемолитическое влияние фенотиазиновых препаратов на прикрепление спектрриновой сети в эритроцитах

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Резюме

Введение: Хлорпромазин, тиоридазин и трифлуоперазин являются фенотиазиновыми препаратами, которые по неизвестному механизму вызывают коллоидно-осмотический гемолиз эритроцитов человека. Для выяснения этого механизма было исследовано влияние этих препаратов на диэлектрическую релаксацию β_{sp} (1.4 МГц) и γ_{1sp} (9 МГц). Было показано, что каждое расслабление снижает силу разрыва специфического мостика, соединяющего сеть спектрина с липидной мембраной. Для релаксации β_{sp} это мостик спектрин-актин-гликофорин С, а для релаксации γ_{1sp} это мостик спектрин-анкирин-полоса 3.

Цель: Выяснить механизмы действия фенотиазиновых препаратов в прегемолитических концентрациях на плазматическую мембрану эритроцитов с помощью сканирующей температурозависимой (термической) дифференциальной диэлектрической спектроскопии.

Материалы и методы: Из свежезабранной крови выделяли эритроциты и 100 μl из них суспендировали в 1 ml изотонического раствора 10 mM NaCl и маннита (рабочая среда), содержащего указанную концентрацию препарата, на 10 мин при 23°C. Обработанные эритроциты выделяли, суспендировали в рабочей среде с гематокритом 0.55 и нагревали (скорость нагрева 1.5° C/min) выше температуры денатурации спектрина (T_A≈49.5°C) с целью получения данных дифференциальной диэлектрической спектроскопии. Комплексный адмиттанс Y*=Y‘+j.Y“ испытуемых суспензий непрерывно измерялся и разделялся на действительную (Y‘) и мнимую (Y“) части с использованием частотно-импедансного анализатора Solartron 1260.

Результаты: В предгемолитических концентрациях каждый препарат ингибировал эти две релаксации, преимущественно релаксацию γ_{1sp}. Результаты можно интерпретировать как сигмовидное действие препаратов на мостик спектрин-анкирин-полоса 3, разрывая его при концентрации непосредственно перед началом массивного гемолиза.

Заключение: Исследование указывает на возможный механизм повреждения эритроцитов после лечения фенотиазиновыми препаратами в предгемолитических концентрациях. Вероятно, это связано с разрушением мостиков между фосфолипидным бислоем и подмембранной спектриновой сетью.

Ключевые слова

диэлектрическая релаксация, лекарственный гемолиз, мембрана эритроцитов