Interaction of Pentoxifylline with Human Erythrocytes. II. Effects of Pentoxifylline on the Erythrocyte Membrane

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The effects of pentoxifylline and other hemorheologically active drugs on the human erythrocyte membrane were examined by means of electron spin resonance spectroscopy. It was observed that the fluidity in the region of the phospholipid head groups in the erythrocyte bilayer was increased by an externally added drug. In this region, membrane fluidity was dependent on the incubation time, suggesting an interaction with membrane proteins. On the other hand, the acyl chain motion in the lower portion of the chain, the hydrophobic end, was reduced in the presence of the drugs. In this case, the acyl chain motion was not time-dependent. These changes of the membrane fluidity at different depths of the membrane induced by the drugs may correlate to the erythrocyte deformability.

Keywords dilazep dihydrochloride; pentoxifylline; trapidil; ESR spectrum; spin label; 5-doxyl stearic acid; 16-doxyl stearic acid; erythrocyte; membrane fluidity

Several drugs, such as dilazep dihydrochloride,[1] pentoxifylline,[2–5] and trapidil,[6] have been used for the treatment of circulatory insufficiencies. These hemorheologically active drugs improve the circulation of blood by increasing the deformability of erythrocytes. In this process, the increase of intracellular adenosine triphosphate (ATP) content affects membrane functions through several mechanisms, which regulate intracellular ionic concentrations, hemoglobin binding to the membrane, exchange of membrane phospholipids, phosphorylation of membrane proteins, *etc.*[7] Three major factors are involved in erythrocyte deformability: internal viscosity, the surface-to-volume ratio and intrinsic membrane properties. The intracellular ATP content can affect all three factors, which are deeply interrelated in a very complex fashion. Membrane deformation, however, can be expressed in terms of three fundamentally independent factors, (i) shear, (ii) expansion, and (iii) bending.[8,9] The changes in (ii) and (iii) induce mainly morphological change, an invagination and/or budding in the erythrocytes. The shear elasticity is an important factor for the change of erythrocyte deformability and is dependent on the membrane fluidity.

Previously, we reported that xanthine derivatives, including pentoxifylline, exhibit two types of binding to erythrocyte ghosts, binding or adsorption to the lipid phase and to other membrane components such as proteins.[10] To clarify further the interaction of pentoxifylline with erythrocyte membrane, we have carried out electron spin resonance (ESR) studies. We have investigated the effects of pentoxifylline on the fluidity at different depths of the membrane by using two kinds of fatty acid spin label compounds.

Experimental

Materials Dilazep dihydrochloride (tetrahydro-1*H*-1,4-diazepine-1,4-(5*H*)-dipropanol bis(3,4,5-trimethoxybenzoate)dihydrochloride monohydrate), pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-xanthine) and trapidil (7-diethylamino-5-methyl-*s*-triazolo[1,5-*a*]-pyrimidine) were gifts from Kowa Co., Ltd., Hoechst Japan, Ltd. and Mochida Pharmaceutical Co., Ltd., respectively. The fatty acid spin label compounds were purchased from Aldrich Chem. Co. In this study, the abbreviations of 5-doxyl stearic acid and 16-doxyl stearic acid, respectively, are used for 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl and 2-(14-carboxytetradecyl)-2-ethyl-4,4-diethyl-3-oxazolidinyloxyl. All other chemicals and solvents used were of analytical grade purity.

Preparation of Spin-Labeled Erythrocyte Suspensions for ESR Measurements Heparinized human blood was obtained from Miyagi Prefectural Red Cross Blood Center. Red cells were washed with 0.9% NaCl to remove the buffy coat and further washed three times with 310 ideal milliosmolarity (mosM) sodium phosphate buffer, pH 7.4.

Spin-labeled erythrocytes (hematocrit of about 90%) were obtained as follows. The washed cells were incubated for 10 min at 37 °C with the spin label, which was previously formed as a thin film on the wall of a flask. The concentration of the spin label in the incubation medium was 2×10^{-4} M. The suspension of spin-labeled erythrocytes for ESR measurements (hematocrit of about 45%) was prepared by adding the same volume of the drug solution.

Preparation of Ghosts The erythrocyte ghosts were prepared by the method of Dodge *et al.*,[11] and resealed by incubating at 37 °C for 20 min in 310 mosM phosphate buffer, pH 7.4. Ghost suspensions were labeled with spin probes in the same way as erythrocytes.

ESR Measurements The spin-labeled erythrocyte suspensions were incubated with xanthine derivatives under various conditions, and then packed in a hematocrit capillary tube (Hyland Co., 1.1 mm inner diameter). The ESR spectra of the packed samples were recorded on a JEOL JES-FE ESR spectrometer (field intensity, 3290 gauss) equipped with a variable-temperature accessory. All spectral parameters were taken from at least three different preparations of labeled erythrocytes.

Results

As shown in Fig. 1, 5-doxyl stearic acid has the nitroxide radical located close at the polar end, whereas 16-doxyl stearic acid has the ring near the hydrophobic end.

The ESR spectrum of 5-doxyl stearic acid in erythrocyte membrane (Fig. 2 inset) is characteristic of the anisotropic motion anticipated for the upper portion of the acyl chains of the membrane lipids (polar end). In Fig. 2, the effects of interaction of drugs with erythrocytes on membrane fluidity are presented in terms of the variation of the convenient order parameter, h_{+1}/h_0, as a function of incubation time. h_{+1} and h_0 represent the peak heights of the low field line and of the central line, respectively. The peak height ratio, h_{+1}/h_0, among the three resonance lines should be influenced by (a) the rate of axial rotation of the spin-label moiety, (b) the rate of tumbling motion of the rotational axis, and (c) the angular amplitude of the tumbling motion.[12] Thus, the peak height ratio can be used as a parameter of membrane fluidity.[12–14] The conventional outer and inner hyperfine splittings, $2T_{\parallel}$ and $2T_{\perp}$, were not applicable in this study because of their small changes. As shown in Fig. 2, dilazep dihydrochloride, pentoxifylline and trapidil had an influence on the acyl chain ordering at

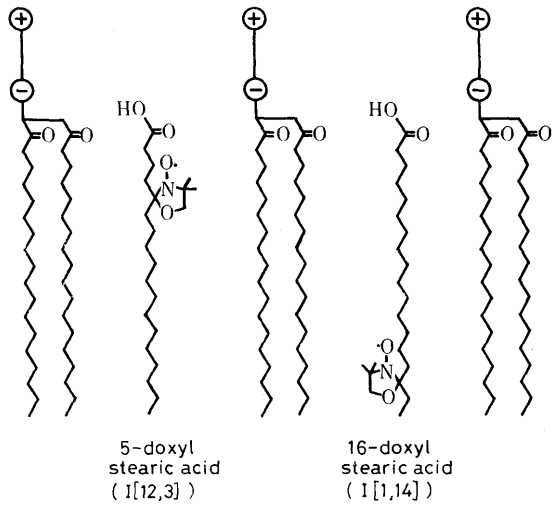

Fig. 1. Spin Labels Used in This Study

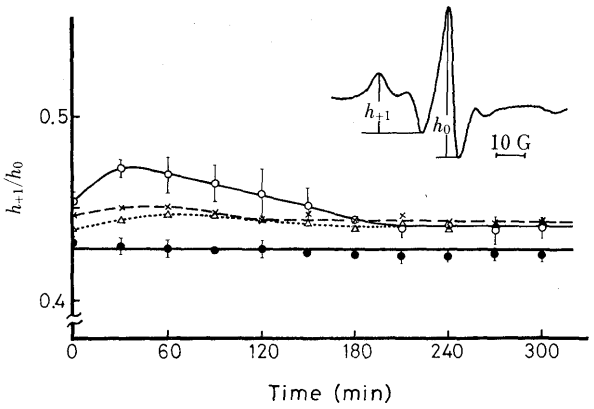

Fig. 2. Variation of h_{+1}/h_0 of 5-Doxyl Stearic Acid in Erythrocytes as a Function of Incubation Time in the Presence of Hemorheologically Active Drugs

—●—, control; —×—, dilazep dihydrochloride; —○—, pentoxifylline; ---△---, trapidil. [Drug] = 5×10^{-5} M. Each point represents the mean ± S.D. of 5 experiments. Inset: ESR spectrum of 5-doxyl stearic acid in erythrocyte membrane. [5-Doxyl stearic acid] = 1×10^{-4} M.

around position 5, indicating that the fluidity of the bilayer near the phospholipid head groups changed during the drug treatments. After the addition of the drugs the values of the parameter were increased and then gradually decreased. The final constant value of the parameter seemed to be nearly equal to its initial value which was obtained immediately after the addition of the drugs.

Figure 3 shows the variation of the parameter when 16-doxyl stearic acid was used as a spin probe. From the spectrum of 16-doxyl stearic acid in erythrocyte membrane shown in the inset, the acyl chain was more mobile at the hydrophobic end than it was near the phospholipid head groups.[15,16] The addition of the drugs, however, reduced the acyl chain motion of this portion.

From these results, it seems that the three drugs used in this study have similar influences on the acyl chain motions both in the upper and lower portions of the acyl chain, but differ in the extent of the effects. Pentoxifylline effectively influenced the fluidity of the membranes. Thus, the effect of pentoxifylline on the erythrocytes was next examined.

In Fig. 4, the changes of the parameter are shown as a function of the pentoxifylline concentration. The initial

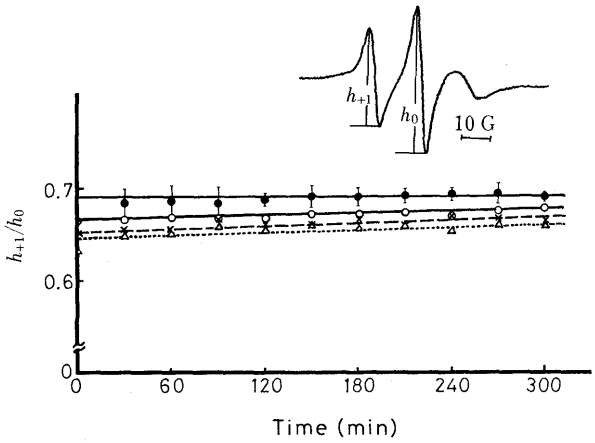

Fig. 3. Variation of h_{+1}/h_0 of 16-Doxyl Stearic Acid in Erythrocytes as a Function of Incubation Time in the Presence of Hemorheologically Active Drugs

—●—, control; —×—, dilazep dihydrochloride; —○—, pentoxidylline; ---△---, trapidil. [Drug] = 5×10^{-5} M. Each point represents the mean ± S.D. of 5 experiments. Inset: ESR spectrum of 16-doxyl stearic acid in erythrocyte membrane. [16-Doxyl stearic acid] = 1×10^{-4} M.

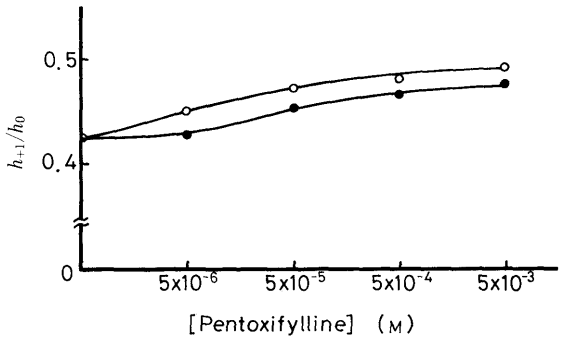

Fig. 4. Variation of h_{+1}/h_0 of 5-Doxyl Stearic Acid in Erythrocytes as a Function of Pentoxifylline Concentration at 37 °C

—●—, without incubation (immediately after addition of pentoxifylline); —○—, after 30 min of incubation. [5-Doxyl stearic acid] = 1×10^{-4} M.

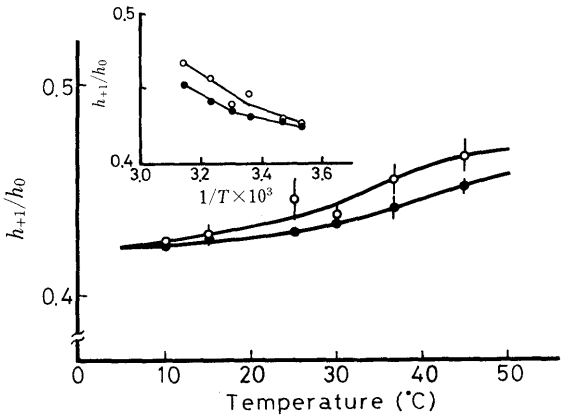

Fig. 5. Variation of h_{+1}/h_0 of 5-Doxyl Stearic Acid in Erythrocytes as a Function of Incubation Temperature

—●—, control; —○—, in the presence of pentoxifylline (after 30 min of incubation). Inset: the same data plotted against the reciprocal of absolute temperature. [Pentoxifylline] = 5×10^{-5} M. [5-Doxyl stearic acid] = 1×10^{-4} M. Each point represents the mean ± S.D. of 3 experiments.

value of the parameter gradually increased as the amount of the drug was increased. The shape of the curve after 30 min of incubation was similar to that immediately after the addition of the drug. These curves correspond to the

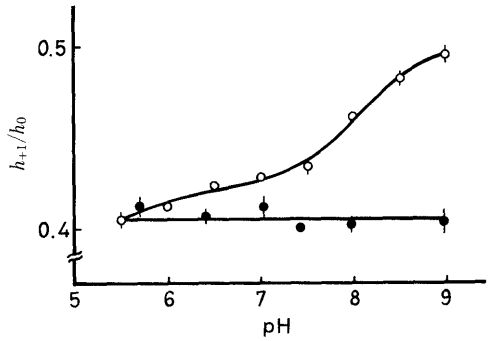

Fig. 6. Variation of h_{+1}/h_0 of 5-Doxyl Stearic Acid in Erythrocytes as a Function of pH in the Presence of Pentoxifylline

—●—, control. [Pentoxifylline] $= 5 \times 10^{-5}$ M. [5-Doxyl stearic acid] $= 1 \times 10^{-4}$ M. The values of h_{+1}/h_0 were estimated after 30 min of incubation at 37 °C. Each point represents the mean ± S.D. of 3 experiments. The solid line is the sum of the two theoretical curves deriving from $h_{+1}/h_0 = a + b/(1 + 10^{pK_1 - pH}) + c/(1 + 10^{pK_2 - pH})$. The values of a, b, c, pK_1, and pK_2 were determined so as to give the smallest mean-square error between the observed (○) and theoretical values. ($a = 0.39$, $b = 0.03$, $c = 0.08$, $pK_1 = 5.7$, $pK_2 = 8.1$).

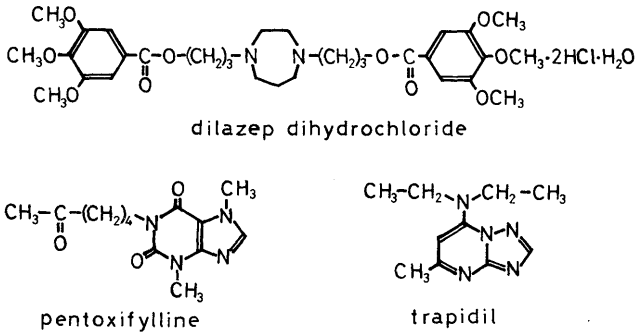

Fig. 7. Structures of Hemorheologically Active Drugs Used in This Study

binding isotherm. The isotherm after 30 min of incubation shifted to the lower concentration side. This indicates that the effect of pentoxifylline on the membrane fluidity was enhanced by the incubation.

Figure 5 shows plots of the change of the parameter as a function of incubation temperature. In the absence of the drug, a transition could be observed at about 37 °C (Fig. 5 inset). Janoff *et al.*[17] also reported that the spin probe 5-doxyl stearic acid revealed a thermally-induced structural transition in the erythrocyte membrane at 37 °C. They also observed a thermotropic transition at 15 °C. However, in this study we could not clearly identify the lower temperature transition. By the addition of the drug, the transition temperature was shifted to about 25 °C.

To characterize further the interaction of pentoxifylline with erythrocyte membrane, we examined the pH-dependence of the parameter at various incubation times. Figure 6 shows the change of the parameter after incubation for 30 min in the presence of pentoxifylline as a function of pH. The value of the parameter increased with increase of pH, showing inflection points at about 5.7 and 8.1. Similar results were obtained when erythrocyte ghosts were used (data not shown). In the absence of the drug, the variation of the parameter was very small. Rigaud *et al.*[18] also reported that no significant change of the ESR spectrum was seen between pH 4 and pH 9 when 5-doxyl stearic acid was used as the probe of erythrocytes. The pH-sensitivity observed here may be attributable to the dissociation of membrane proteins which could interact with pentoxifylline. The values of the parameter for the control in Fig. 6 are different from those in Fig. 2. This variation may be due to the fact that the blood samples were collected on different days.

Discussion

The present study was motivated by the report of Ogiso *et al.*[16] dealing with the effect of chlorpromazine on membrane fluidity. They reported that the fluidity of the fatty acid chains at about ten carbons removed from the bilayer surface was increased by chlorpromazine treatment. In the present study, we examined the effects of three hemorheologically active drugs (dilazep dihydrochloride, pentoxifylline and trapidil) on the fluidity at different depths in the erythrocyte membranes.

As can be seen from Figs. 2 and 3, the effect of the drugs on the polar end is very characteristic. The effects on the fluidity in the upper portion of the membrane can be classified into two cases (Fig. 2), *i.e.*, dependent on the incubation time, and independent of it. The values of the parameter in the first case increased with incubation time and then gradually decreased to reach constant values. The maximal values of the parameter were observed at 30—60 min of incubation. In the second case, the values of the parameter were constant, which may correspond to the final values on prolonged incubation or to the initial values just after the addition of the drugs. On the other hand, at the hydrophobic end the change in the parameter was monotonous. These results suggest that these drugs interact with erythrocytes in at least two different binding modes. Previously, we observed two classes of binding sites for pentoxifylline, binding to lipid and protein phases.[10] The two binding types observed in this study should correspond to those previously observed. In the presence of caffeine, theobromine or theophylline, no time-dependent parameter change was observed (data not shown). These xanthine derivatives also have two types of binding to erythrocyte ghosts.[10] Further, in the liposome suspension which was formed from egg phosphatidylcholine, pentoxifylline did not show the time-dependent change. Therefore, it is reasonable to consider that the time-independent interaction should be adsorption on the lipid phase and that the time-dependent interaction represents the interaction of pentoxifylline with membrane proteins accompanying a biochemical reaction.

The addition of pentoxifylline shifted the transition temperature to a lower value. Janoff *et al.*[17] assumed that the transition results from the association with protein and lipid. The alteration in thermal transition observed in this study indicates that pentoxifylline affects some specific membrane domain such as sites of lipid–protein interaction. It seems that this specific domain is a hydrophobic region of β-structure of the membrane proteins.[10]

Although these drugs are not similar in their chemical structures (Fig. 7), it should be stressed that they have similar effects on the membrane fluidity. That is, when these drugs were externally added to erythrocyte suspension, the fluidity of the erythrocyte bilayer near the outer surface (the upper portion) was increased in a time-dependent manner, whereas the lower portions became more rigid. The change

of the membrane fluidity induced by these drugs results in an enhancement of the shear elasticity of the membrane surface, altering the erythrocyte deformability. The increased rigidity at the lower portion of the membrane seems not to have such a marked effect on the shear elastic change.

Concerning the erythrocyte deformability, an improvement of internal viscosity is also required. Although the internal viscosity was not examined in the present study, pentoxifylline was shown to cause a dose-related decrease in whole blood viscosity.[19,20] It was postulated that such a viscosity change was induced *via* an increase in erythrocyte ATP content.[5] From the results of present study using pentoxifylline, it was suggested that some biological processes participate in the enhancement of the fluidity at the upper portion of the membrane. Since the molecular ordering of the fatty acid chains depends significantly on the type of polar group,[15] this biological process may include some changes of polar groups. The inflection points shown in Fig. 6 should correspond to the pK values of the proteins involved in the postulated biological process. It is uncertain, however, whether this biological process is the same as that inducing the change of ATP content or of polar groups. The influences of changes of polar groups on the parameter h_{+1}/h_0 will be reported in the following paper.

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