Interaction of Pentoxifylline with Human Erythrocytes. I. Interaction of Xanthine Derivatives with Human Erythrocyte Ghosts

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The interaction of pentoxifylline and other xanthine derivatives with human erythrocyte ghosts was studied. By fluorescence spectroscopy it was found that xanthine derivatives have two modes of binding to erythrocyte ghosts. One is a high-capacity binding to erythrocyte membranes. It seems that the 5-oxohexyl side chain of pentoxifylline is important for tnis. The second type may be a binding to proteins on the membranes and is specific for pentoxifylline and caffeine. From the circular dichroism spectra, it was presumed that the second binding mode of pentoxifylline occurs at hydrophobic regions of β-structure of the membrane proteins. The relative high specificity in the interaction of pentoxifylline with erythrocytes should be related to its unique physiological activity on erythrocytes.

Keywords erythrocyte ghost; xanthine derivative; pentoxifylline; fluorescence spectroscopy; binding parameter; binding capacity

The blood flow in vessels is partly dependent on the flexibility of the erythrocyte membrane and the fluidity of the intracellular contents. 3,7-Dimethyl-1-(5-oxohexyl)-xanthine (pentoxifylline), used as a drug to improve the flow properties of blood, has the ability to deform erythrocytes by increasing their cellular adenosine triphosphate (ATP) content.[1-5] Nakao *et al.*[6] firstly observed that the shape of the cells is dependent on ATP concentration. Weed *et al.*[7] also found that ATP content correlated with red blood cell deformability. They proposed that the erythrocytes undergo ATP-calcium-dependent sol–gel change at the interface between the membrane and the cell interior, and that the sol–gel balance determines membrane deformability. The characteristic physiological activities of pentoxifylline may be related to the interaction with erythrocytes.[8] The activity of many drugs is related to their abilities to combine with specialized functional proteins. Further, the characteristic properties of biological membranes depend on the conformational states of these proteins being changed cooperatively by interactions among the proteins and with specific ligands. Among possible mechanisms for the change of deformability of erythrocytes caused by drugs, changes in conformation or association of skeletal membrane proteins seem most plausible.[9,10]

The aim of the present study was to investigate a possible effect of pentoxifylline on membrane fluidity. In this paper, the bindings of pentoxifylline and other xanthine derivatives to erythrocyte ghosts were measured by fluorescence and circular dichroism (CD)spectroscopy to evaluate whether pentoxifylline has a characteristic interaction with red cells.

Experimental

Materials Pentoxifylline was a gift from Hoechst Japan, Ltd. Caffeine was obtained from Kanto Chemical Co., Inc. Theobromine and theophylline were from Nakarai Chemicals, Ltd. They were recrystallized from water except for pentoxifylline. All other chemicals were of analytical grade. Water was deionized and doubly distilled from an all-glass system.

Preparation of Erythrocyte Ghosts The erythrocyte ghosts were prepared according to the method of Dodge *et al.*[11] from fresh heparinized human blood. Resealed ghosts were prepared from open ghosts by suspending them in isotonic phosphate buffer (310 mosM, pH 7.4) and incubating at 37 °C for 10 min. The protein content of the ghost suspension was determined by measuring the mean residue ellipticity at 223 nm. The mean residue weight of 130 was used to compute ellipticities. It was found that the ghost suspension of 1×10^{-3} M contained about 0.13 mg protein per ml. It should be noted that in this study the ghost concentration is represented by the amino acid residue concentration.

Fluorescence Spectroscopy Fluorescence measurements (excited at 280 nm) were made with a Jasco FP-550 recording spectrofluorometer at room temperature. The samples for spectroscopic measurements were prepared by adding the drug solution to the ghost suspension. The spectra were measured within an hour after the addition. The absorption and fluorescence spectra of the ghost suspension used in this study did not show any hemoglobin bands, and during the binding experiments, the absorption intensities of the ghost suspension were apparently linear. It seems that the ghost suspension was essentially free of the gross distortions caused by absorption flattening and light scattering. Further, these results indicate that the hemoglobin content of the ghosts was significantly small, and that fluorescence arising from hemoglobin and inner filter effect could be neglected. Thus, the recorded readings were used without any correction.

CD Spectra The CD spectra were measured with a Jasco J-400X spectropolarimeter equipped with a data processor. The measurement was carried out at room temperature. When optical measurements are made for turbid suspensions such as erythrocyte membranes, it is necessary to take into account the distortions arising from the particulate nature of the samples.[12,13] Concerning the ghost suspensions used in this study, no pathlength dependence was observed within a cell length of 1—5 mm. This indicates that differential scattering could be neglected and that the observed CD spectra of the ghost suspensions were not significantly altered by optical artifacts.[12,14] The observed CD was expressed in terms of molar ellipticity, $[\theta]$, in deg·cm^2/dmol.

Determination of Parameters for Binding of Xanthines to Ghosts To determine the saturation bindings of xanthines (F_{max}) to ghosts, the fluorescence emission spectra were measured at a fixed concentration of xanthine solution with various ghost membrane concentrations. By extrapolating a plot of 1/(relative fluorescence) ($1/F$) *vs.* 1/(ghost membrane concentration) to the y-axis, F_{max} was determined. The value of x, which was taken to be the ratio of the observed fluorescence at a given membrane concentration to F_{max} (F/F_{max}), was also determined from the same data. To determine the binding of drug molecules to independent sites on proteins, the data were plotted according to the equation of Klots *et al.*[15] The number of drug binding sites per ghost, n, and the average apparent association constant for the drug–ghost complex, K, were determined by the use of Eq. 1:

$$[\text{ghost}]/x[\text{drug}] = 1/n + [Kn(1-x)[\text{drug}]]^{-1} \quad (1)$$

where [ghost] is the total ghost concentration, [drug] is the total drug concentration, and x is the fraction of the drug bound.

Results and Discussion

Interaction of Xanthine Derivatives with Erythrocyte Ghosts In order to characterize the interaction of pentoxifylline with erythrocytes, we measured fluorescence spec-

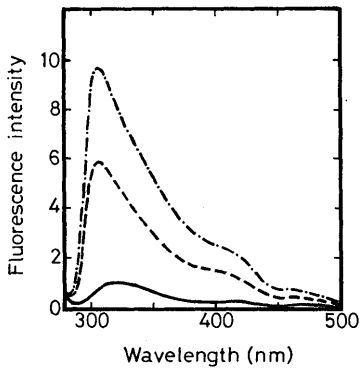

Fig. 1. Fluorescence Spectra of the Pentoxifylline-Ghost System

[Pentoxifylline] = 1×10^{-3} M. ——, pentoxifylline alone; — —, in the presence of ghosts (2.5×10^{-4} M); —·—, in the presence of ghosts (5.0×10^{-4} M).

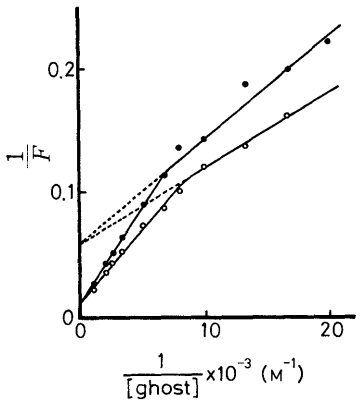

Fig. 2. Double Reciprocal Plot of Fluorescence Change and Ghost Concentration

—○—, [pentoxifylline] = 5×10^{-4} M; —●—, [pentoxifylline] = 1×10^{-3} M.

tra derived from xanthine derivative–erythrocyte ghost systems. Figure 1 shows the fluorescence spectra resulting from mixtures of various amounts of ghost suspension with a fixed amount of pentoxifylline. Pentoxifylline itself exhibits fluorescence emission maxima at arount 470, 410 and 325 nm. The fluorescence was increased by the addition of the ghost suspension. Such increases in fluorescence intensity were observed with caffeine, theophylline, theobromine and pentoxifylline in decreasing order. This is due to an increase in quantum yield of the derivatives added. Such spectral changes were also observed upon ethanol addition in the absence of ghost membranes. In this case, however, the fluorescence increased with theobromine, theophylline, caffeine, and pentoxifylline in decreasing order. These results suggest that the drugs are transferred to a less polar environment than water, and that the drugs interact not only with lipid phase but also with other membrane components such as proteins.

Characteristic Binding of Pentoxifylline For the analysis of fluorescence data in terms of the binding parameters, the intensities at 410 nm were used, because erythrocyte ghosts have some fluorescence emission at 333 nm, due to tryptophan residues in the protein. The binding parameters of the drug-ghost system were calculated from double-reciprocal plots to evaluate whether a change in quantum yield contributed to the increase of pentoxifylline fluorescence (Fig. 2). As can be seen, the quantum yield of bound

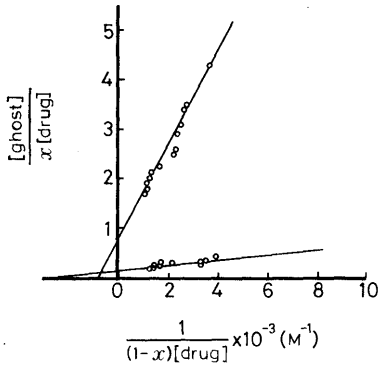

Fig. 3. Klotz Plots for Binding of Pentoxifylline to Ghosts

($Y = 0.97X + 0.73$, $r = 0.977$; $Y = 0.05X + 0.14$, $r = 0.837$). The data are the same as in Fig. 2.

TABLE I. Number of Binding Sites (n) and Association Constants (K) for the Binding of Xanthine Derivatives to Ghosts

Drugs	Class I		Class II	
	n	K	n	K
Caffeine	13.4	1.2	0.7	1.7
Pentoxifylline	7.0	2.6	1.4	0.8
Theobromine	16.9	1.3	4.0	0.3
Theophylline	16.2	1.3	3.8	0.3

The binding parameters given in the table are the mean values of 2 experiments. n, mol/mol; K, $\times 10^{-3}$ M^{-1}.

pentoxifylline was increased in the presence of ghost membranes. The intercept on the ordinate give F_{max}, which is a relative measure of the quantum yield of bound pentoxifylline.[16] The plots of the reciprocal of the ghost concentration are linear, and undergo biphasic changes with two intercepts. These two intercepts seem to be independent of pentoxifylline concentration. This means that the fluorescence intensity of the pentoxifylline–ghost complexes is apparently constant. These results suggest the presence of two modes of binding. Similar observations were obtained with other xanthine derivative–ghost systems.

These data were plotted according to Eq. 1 (Fig. 3). The calculated binding parameters, n and K, are given in Table I. The experimental results show that xanthine derivatives have two modes of binding to erythrocyte ghosts. Elferink[16] has already reported that erythrocytes possess two classes of binding sites for chlorpromazine and its quaternary analogues. Many other drugs also seem to have more than one mode of binding to erythrocytes. Xanthine derivatives were bound to the ghost membranes mainly through hydrophobic interaction. Several authors have postulated that both the lipid and the protein phases are important binding domains in erythrocyte membranes.[16]

In the xanthine derivative–ghost systems, it also seems that the drugs are bound to the protein as well as to lipid of the membrane. The difference between the two modes of binding is characterized by the binding capacity ($n \times k$). In this study, since the ghost concentration was expressed on a molar protein basis, the number of binding sites corresponds to that on proteins in the ghost membranes. In Table I, class I corresponds to the binding or adsorption to the lipid phase not to some particular proteins, because of

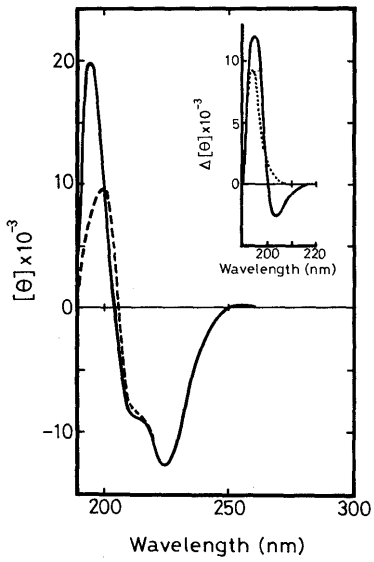

Fig. 4. Effect of Pentoxifylline on the CD Spectrum of Erythrocyte Ghosts

——, (a) erythrocyte ghosts; — —, (b) erythrocyte ghosts in the presence of pentoxifylline. [Pentoxifylline] = 5×10^{-4} M. [ghost] = 1×10^{-3} M. The inset shows the difference CD spectrum between (a) and (b) (· · · ·, caffeine–ghost system).

its rather large binding capacity. It should be noted that the binding parameters for pentoxifylline are different from those of the other derivatives. That is, the number of binding sites is relatively small and the association constant is large. In the fluorescence spectra, the enhancement of the pentoxifylline band by ghosts was the smallest. Since the fluorophore portion of the derivatives is common, the substituent groups are responsible for these differences. This implies that the 5-oxohexyl side chain at the N-1 position in pentoxifylline makes a major contribution to the binding to ghost membranes. This side chain should be located in the lipid phase of the membranes.[17] The small value of n suggests that the binding of pentoxifylline to membrane is specific.

On the other hand, for class II which may correspond to interaction of drug with protein, it can be postulated that pentoxifylline and caffeine have specific interactions, because the number of binding sites is close to 1 in each case and the association constants are larger than those of other derivatives. The magnitude of the association constants evaluated in the xanthine derivative–ghost systems is about ten times smaller than that in the chlorpromazine–ghost system.[16] Although the value of parameters is strongly influenced by small variations in the experimental conditions,[18] the difference in the magnitude of the association constant between xanthine derivatives and chlorpromazine systems indicates an anomalous perturbation effect of chlorpromazine on the membranes.[19,20] Compared with chlorpromazine, the xanthine derivatives studied do not have a strong affinity for erythrocyte membranes. The derivatives, nevertheless, can change several physicochemical properties of erythrocytes, such as the surface charges and elastic properties.[21,22] The characteristic actions of pentoxifylline on erythocyte membranes improving erythrocyte deformability characteristics should be primarily due to its specific interactions with membrane components of erythrocytes. Two classes of binding sites for pentoxifylline, binding to lipid and protein, were also observed in electron spin resonance (ESR) experiments.[23]

The effect of externally added pentoxifylline on the membrane proteins was also examined by CD spectroscopy. Figure 4 shows the CD spectra of erythrocyte ghosts in the absence and presence of pentoxifylline. The difference CD spectrum illustrated in the inset is similar to that of antiparallel β-structure.[24] Other xanthine derivatives did not show such a large change in the CD spectrum. These results indicate that pentoxifylline molecules bind specifically to the hydrophobic β-structure regions of the membrane proteins. Thus, it can be said that pentoxifylline interacts specifically with erythrocyte membranes.

Recently, Thao Chan *et al.*[8] examined the effects of xanthine derivatives on erythrocyte deformability by using filtration through polycarbonate filters. They reported that only pentoxifylline had a significant effect on erythrocyte deformability. Their results correspond to the present findings. The specific interaction of pentoxifylline with membrane components is an essential step in the process of deformability improvement.

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