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Effects of Calcium Channel Blockers on Human Erythrocyte Ghost Membranes

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The effects of calcium channel blockers (CAB's), verapamil, diltiazem and nicardipine, on erythrocyte ghost membranes have been studied. Using the fluorospectroscopic method, it was observed that the fluidity of the inner layer of ghost membranes was increased with an increase of drug concentrations but did not any changes in the fluidity of the outer layer. These drugs showed protective effect against hypotonic hemolysis of erythrocytes. Thus, the expansion of surface area in response to corpuscular volume of erythrocytes in the presence of CAB's is seemed to play an important role in protecting hypotonic hemolysis of erythrocytes.

Key words : Calcium channel blockers, Human erythrocyte ghosts, Membrane fluidity, Hypotonic hemolysis, Membrane stabilization

INTRODUCTION

A number of calcium channel blockers (CAB's) have been used for treating a variety of cerebro- and cardio-vascular diseases including hypertension, artrial arrhythmia and angina pectoris. It is impotant to understand the mode of action of these agents in cardiac and vascular smooth muscle. So the mode of action by CAB's in smooth-muscle, cardiac muscle and other organs has received considerable attention and has been recently reviewed (Henry, 1980; Kawai *et al.*, 1981; Van *et al.*, 1981; Brodsky *et al.*, 1982). The result represent that CAB's exert their actions primarily at the potential-dependent calcium channels located in the plasma membranes. Schanne *et al.* (1979) have postulated that the crucial mechanism of calcium mediated cell death was massive influx of calcium ions following membrane damage. Peck & Lefer (1981) reported that CAB's protected the liver during hypoxia and that this protection might have stemmed from its inhibition of Ca influx which had been linked to irreversible cell death. And Abe *et al.* (1991) studied the effect of CAB's on erythrocyte membranes and showed their protective effects against hypotonic hemolysis. Such the protective effects of CAB's on biomembranes seemed to be caused through cytoprotective effects such as membrane stabilization. Mobility of molecules in biomembranes is as whole expressed as membrane fluidity. The term, membrane fluidity, is usually used in a more qualitative sense, generally meant to be a measure of the resistance of various type in the membrane (Genesis, 1989).

In this experiment, the fluidity of biomembranes by CAB's was studied using two fluorescence probes, 1, 6-diphenyl-1,3,5-hexatriene and 1,3-di(1-pyrenyl)propane. It is the purpose of this study to investigate the mechanism of the protective effect of CAB's on biomembranes by observing the motions of these probes incorporated in the bilayer of biomembranes.

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MATERIALS AND METHODS

Materials

Verapamil, diltiazem, nicardipine, 1,6-diphenyl-1,3,5-hexatriene (DPH), and white mineral oil were purchased from Sigma Chem. Co. (St. Louise, MO. U.S.A). 1,3-Di(1-pyrenyl)propane (py-3-py) was a gift from Dr. Yeun, College of Dentistry, Pusan National University. All other reagents were of analytical grade. Phosphate buffered saline (pH 7.4) was made of monobasic potassium phosphate and dibasic sodium phosphate, and added sodium chloride.

Preparation of ghost membranes

Human erythrocytes were obtained by centrifugation at 1,260 rpm for 15 min at 4°C from the freshly drawn blood of normal donors. After removing the plasma water and buffy layer containing

leucocytes, the packed erythrocytes were washed three times by centrifugation at 3,000 rpm for 15 min at 4°C with isotonic buffer solution composed of 140 mM NaCl containing 10 mM phophate buffer (pH 7.4, 285 mOsM phosphate beffered saline, PBS).

Erythrocyte ghost membranes were prepared according to the method of Lee *et al.* (1994). 1 ml of the washed erythrocytes was hemolyzed by the addition of about 30 ml of a hypotonic solution composed of 10 mM phosphate buffer (pH 7.4, 20 mOsM phosphate buffer, PB). The hemolyzed sample was centrifugated at 6,000 rpm for 30 min at 4°C and to remove water soluble fragments this process was repeated two or three times. The resulting sample was resuspended in PBS containing 0.1 mM of $MgCl_2$ and incubated for 30 min at 37°C with slightly shaking to reseal the open erythrocyte ghost membranes by previously conducted hemolysis (Kahana *et al.*, 1991). Resealed erythrocyte ghost membranes were harvested by centrifugation at 4,500 rpm for 30 min. The resealed ghost membranes were stored at 4°C and used for experiments within 2 days.

Measurement of hemolytic activity and inhibition rates

Whole blood was freshly drawned from normal donors and human erythrocytes were collected by centrifugation at 1,000 rpm for 3 min. The buffy coat was discarded and the cells were washed three times with PBS by centrifugation. The volume of erythrocytes was measured and reconstituted as a 1 v/v % erythrocyte suspension with PBS. The erythrocyte suspension was incubated in a solution containing each of various concentrations of CAB's for 20 min at room temperature. The reaction mixture was centrifugated for 3 min at 1,000 rpm and the supernatant was measured at 540 nm of UV-spectrophotometer. The hemolytic activity by CAB's was calculated by comparing with a sample showing 100% hemolysis, treated in hypotonic solution without CAB's. Hemolysis inhibition of erythrocytes by CAB's was measured in hypotonic solution of 10 mM phosphate buffer. Portions (3 ml) of hypotonic solution containing each of various concentrations of CAB's were added to 20 ul of the 1 v/v % erythrocyte suspensions. The concentrations of CAB's were used from 10^{-8} to 10^{-3} M. The mixtures were incubated for 10 min at room temperature and centrifugated for 3 min at 1,000 rpm. The absorbance of the supernatant fluid was measured at 540 nm. The percentage of hemolysis inhibition by CAB's was calculated according to Eq. 1 (Abe *et al.*, 1991).

$$\%\text{Inhibition} = 100 - \frac{\text{ABS of test sample}}{\text{ABS of control sample}} \times 100 \quad \text{Eq. 1}$$

where ABS means the absorbance of UV-spectrophotometer.

Fluorescence polarization measurements

The fluorescence probe, DPH, was dissolved in tetrahydrofuran and 0.5ul of tetrahydrofuran solution was added directly to the ghost suspension containing 100 μg protein of ghost membranes per ml of PBS. The final probe concentration was 1 μg of DPH/100 μg of membrane protein. The suspension containing DPH was incubated, shaking once in a while, in the dark for 30 min at 37°C. Then, CAB's were added to the suspension. The blank was prepared under the same condition without probe to serve as a control of the fluorescence measurement. The depolarization technique of fluorescence probe applied to membrane systems was presented in detail by Schinitzky *et al.* (1971). And the use of DPH as a fluorescence probe, that was specific to the hydrocarbon region of lipid bilayers of biomembranes, was discussed in detail elsewhere (Lentz *et al.*, 1976 a, b; Kawato *et al.*, 1977).

The fluorescence intensity of DPH incorporated in ghost membrane was measured at various concentrations of CAB's from 10^{-8} to 5×10^{-3} M. The excitation and emission wavelengths of DPH was 362 and 430 nm, respectively. The fluorescence polarization (P) was obtained from intensity measurements using Eq. 2.

$$P = \frac{I_{vv} - I_{hv} \times G}{Ivv + I_{hv} \times G} \quad \text{Eq. 2}$$

where G is a grating factor of the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the vertical to horizontal components when the exciting light is polarized in the horizontal direction. Ivv and Ihv are the intensities measured when the polarizer and analyzer prisms are in the vertical or horizontal positions, repectively.

Measurement of excimer-to-monomer fluorescence intensity ratio

The incorporation of py-3-py was carried out by adding aliquots of a stock solution of 5×10^{-5}M in ethanol to the ghost suspensions (100 μg of ghost membrane protein/ml of PBS), so that the final probe concentration was less than 5×10^{-7}M (Zachariasse *et al.*, 1982; Almeida *et al*, 1982). The suspension was incubated at 4°C for 16 hr under gently stirring with magnetic stirrer. Blanks, prepared under identical conditions without py-3-py, were used as controls for the fluorometric measurements. CAB's, regulating its concentration from 10^{-8} M to 5×10^{-3} M, were added

directly to ghost membranes suspended in PBS. The measurements of fluorescence intensities of py-3-py incorporated in ghost membranes were performed with SFM 25 spectrofluorometer (Kontron, Model 25, Swiss) equipped with a polarizer and a thermostatic cell holder. Oxygen was removed by bubbling pure nitrogen through the suspensions for 15 min prior to the fluorescence measurements. The excimer-to-monomer fluorescence intensity ratio(I'/I) was calculated from 430nm to 362nm signal ratio (Zachariasse *et al*, 1980). The excitation wavelength was 338 nm.

Microviscosity of ghost membranes

I'/I values of py-3-py and P values of DPH in the ghost membranes could be translated into viscosity values by comparing these values with those measured in an appropriate solvent of known macroscopic viscosity. For this purpose, white mineral oil was chosen as a reference system. Py-3-py was incorporated into white mineral oil with the same manners as performed in the erythrocyte ghost membrane suspensions, and I'/I was measured using SFM 25 spectrofluorometer at the temperature range of 0°C to 40°C. Fluorescence polarization (P) of DPH incorporated in white mineral oil was also measured at the same range of temperature. The microviscosities of white mineral oil were determined at the temperature range of 0°C to 40°C using Physica rheometer with sensor Z2 (Rheolab SM-HM, German). The calibration curves of $\ln\eta$ (microviscosity) versus I'/I or P values were obtained from both of plots of temperature versus microviscosity of mineral oil and temperature versus I'/I or P of mineral oil. And the microviscosity of ghost membranes was evaluated from the calibration curves of $\ln\eta$ versus I'/I or P of ghost membranes.

RESULTS AND DISCUSSION

Hemolytic activity and inhibition rates of CAB's on hypotonic hemolysis

The hemolytic activities of CAB's were shown in Fig. 1. None of these compounds exhibited a hemolytic activity at the concentrations lower than 10^{-3} M, but diltiazem showed slightly hemolytic activity at the concentration over 10^{-2} M. Verapamil and nicardipine were not used over 10^{-4} M because of their poor solubilities.

Inhibition rates of CAB's for hemolysis of erythrocytes were represented in Fig. 2. All of three compounds showed strong stabilizing effect against hypotonic hemolysis of erythrocytes. The inhibition rates of diltiazem, nicardipine and verapamil were about 15, 25 and 28% at 10^{-5} M, respectively. As

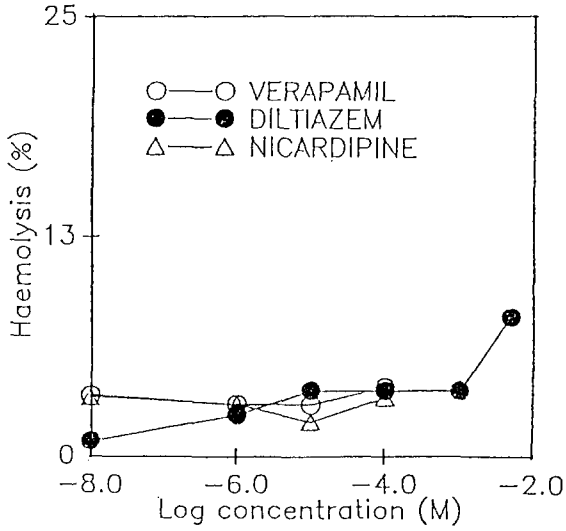

Fig. 1. Hemolytic activity of calcium channel blockers on human erythrocytes at 20°C

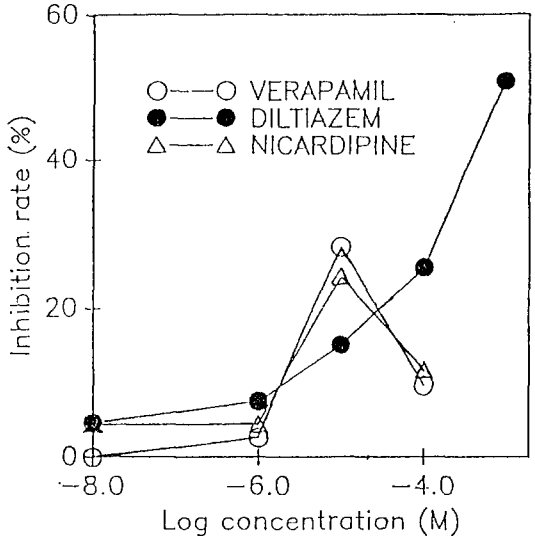

Fig. 2. Protective effects of calcium channel blockers against hemolysis of human erythrocytes in hypotonic solution at 20°C

shown in Fig. 2, the inhibition effect of diltiazem was increased with an increase of its concentration, but the effects of nicardipine and verapamil were contrarily decreased in the concentrations higher than 10^{-5} M, that was a concentration showing their maximum inhibition effects. More investigation was needed on the reason why they had maximum inhibition rates.

Fluidity of ghost membranes; fluorescence polarization method

Fluorescence polarization (P) has been utilized to measure molecular rotational diffusion (Shinitzky *et al.*, 1971; 1076; 1978). When applied to biological

membranes, the rotational motions of some of the probes have often been compared to the measurements made in oils of known viscosity, and quantified in terms of "microviscosity". DPH is the most widely used probe. In fluorescence polarization, polarized light preferentially excites those fluorophores whose molecular axes are orientated in a particular direction with respect to the plane of polarization. If the fluorophore remains immobile during its excited life time (10^{-8} sec), then the fluorescent light will also be highly polarized. However, if the fluorophore rotates during its fluorescent life-time, then the resulting fluorescence will become less highly polarized. The polarization of fluorescence thus acts as a convenient index of the extent of the molecular rotation during its excited life-time.

The effects of CAB's on the fluidity of ghost membranes were investigated by measurements of the fluorescence polarization of DPH incorporated in lipid bilayer of ghost membranes at 20°C. As represented in Fig. 3, the P values of ghost membranes did not change under the concentration range of 10^{-8} to 10^{-6} M. But P values were decreased with an increase of drug concentration from 10^{-6} to 10^{-3} M. It means that the fluidity of ghost membranes increases with an increase of drug concentration. Then, over the concentration of 10^{-3} M, the reversal point appeared with an increase of diltiazem concentrations. Even if the reversal point of verapamil was not shown in Fig. 3, it was also observed through the experiments repeated. In the case of diltiazem, the fluidity of ghost membranes was contrarily decreased at the concentration higher than 10^{-3} M. The reason why the decrease of the fluidity was happened in the case of diltiazem could not be found in this experiment.

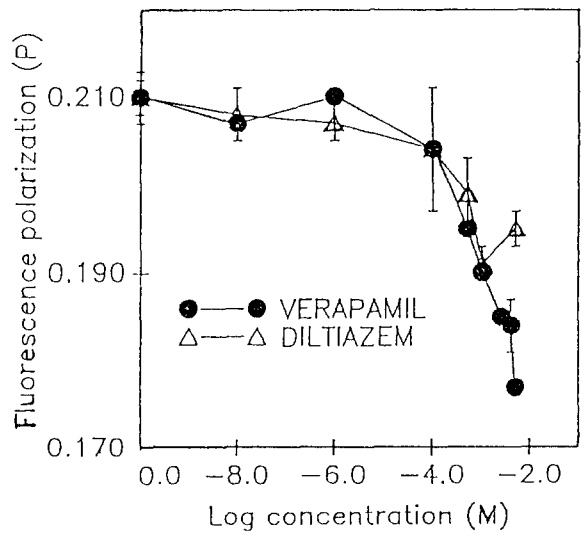

Fig. 3. Effects of verapamil and diltiazem on the fluorescence polarization (P) of DPH incorporated in erythrocyte ghost membranes at 20°C.

Fluorescence intensity ratio

As previously mentioned (Lee *et al.*, 1994), intrmolecular excimer formation of py-3-py has been used to study the fluidity changes of boimembranes. Intramolecular excimer formation, due to association of an excited and an unexcited aromatic group incorporated in the same molecule, can be described by the excimer to monomer fluorescence quantum yield ratio, Φ'/Φ, which is proportional to the monomer and excimer fluorescence intensities, I and I', respectively (Zachariasse *et al.*, 1980; 1982).

$$I'/I = \Phi'/\Phi = \frac{K_f^1}{K_f} \quad Ka\tau_o$$

where K_f (monomer) and K_f^1 (excimer) are the fluorescence rate constants, Ka and τ_o are the rate constant for excimer association and the excimer life time in the absence of excimer dissociation, respectively. Especially, it has been found with py-3-py that, in the low temperature and in highly viscous media, I'/I decreases linearly with increasing viscosity. This makes it possible to use py-3-py as a probe to determine effective viscosities in viscous media (Zachariasse, 1978).

Fluorescence measurements at 4°C, as a function of time after probe incorporation, shows that the excimer to monomer fluorescence intensity ratio reaches a constant value after approximate 10 hr (Lee *et al.*, 1994). Therefore, the incorporation time of 16 hr reflects the time necessary for the complete transfer of the probe molecule from the aqueous phase into the biomembranes. Fig. 4 shows that the values of I'/I have not changed at the concentration under 10^{-3} M of verapamil and diltiazem, but decreases suddenly at the concentration over 10^{-3} M. This reflects that the rapid decrease of I'/I at 10^{-3} M is very similar with the reversal point of the fluorescence polarization measurements. I'/I and P values reflect the fluidity of the outer layer (head group) and the inner layer (acyl chain) of biomembranes, respectively. Accordingly, even if there were not any changes in the fluidity of ghost membranes from excimer to monomer fluorescence intensity ratio measurements until drug concentration reached to 10^{-3} M, it was suggested that, from the decreases of the fluorescence polarization, the fluidity of the inner layer of biomembranes increased. Fig. 5 shows the effect of nicardipine on two probes, DPH and py-3-py, incorporated in ghost membranes over the concentration range of 10^{-6} to 10^{-3} M. It means that nicardipine should be used under 10^{-6} M, because of its low solubility in buffer solution (pH 7.4) and quenching effect of two probes in its high con-

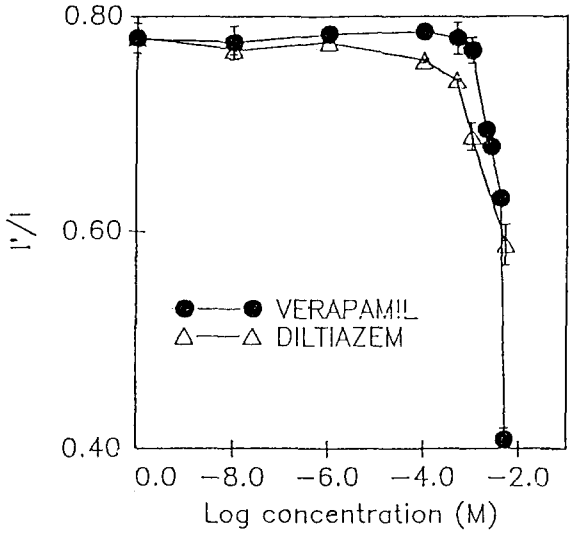

Fig. 4. Effects of verapamil and diltiazem on the excimer to monomer fluorescence intensity ratio of py-3-py incorporated in erythrocyte ghost membranes at 20°C. The intensities of I' and I were determined at 480 and 379 nm, respectively.

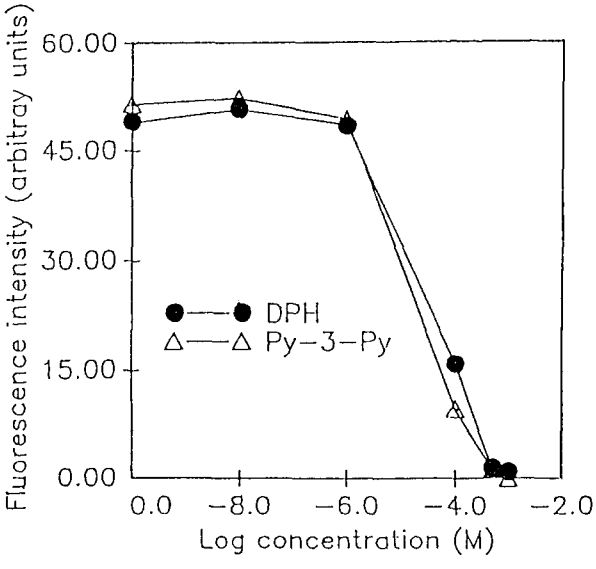

Fig. 5. Effects of nicardipine on the fluorescence intensity of DPH and py-3-py incorporated in erythrocyte ghost membranes at 20°C

centration. From fluorescence determinations, it is confirmed that the fluidity of biomembranes increases with an increase of CAB's concentration, though their effective mechanisms are mutually different.

Microviscosity of ghost membranes by CAB's

P and I'/I values are determined in white mineral oil as a function of temperature and represented in Fig. 6 and 7. As can be seen from Fig. 6 and 7, P values and viscosity (η) decrease with an increase of

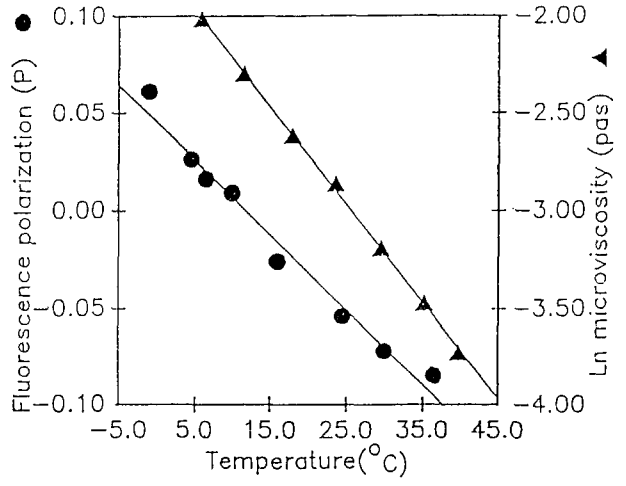

Fig. 6. Microviscosity and fluorescence polarization (P) of DPH observed in white mineral oil as a function of temperature

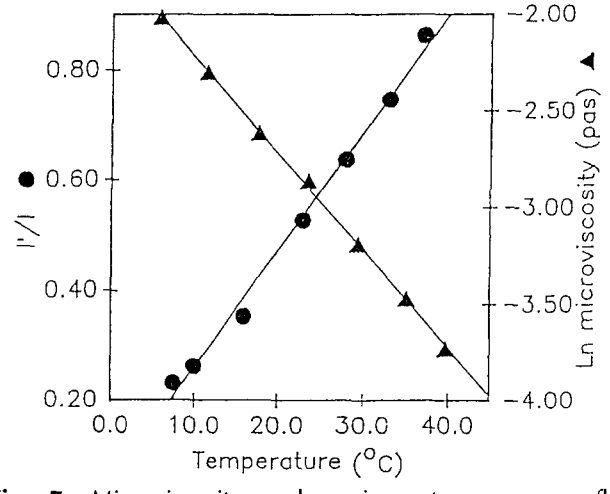

Fig. 7. Microviscosity and excimer to monomer fluorescence intensity ratio (I'/I) of py-3-py measured in white mineral oil as a function of temperature

temperature while I'/I values increase with increasing temperature. In Fig. 8 and 9, the plots of logη versus P or logη versus I'/I are drawn by using the equations obtained from two curves in Fig. 6 and 7, respectively. The microviscosity of ghost membranes is determined from the calibration curves in Fig. 8 and 9 over the concentrations used in this experiment. These results are shown in Fig. 10 and 11.

Sheetz and Singer (1974) have proposed that membranes, whose proteins and polar lipids are distributed asymmetrically in the two halves of the membrane bilayer, can act as bilayer couples, i.e., the two halves can respond differentially to a pertubation. Such pertubation can result in the expansion of one layer relative to the other, thereby producing a curvature change of the membrane. This hypothesis has been applied to suggest a detailed molecular mechan-

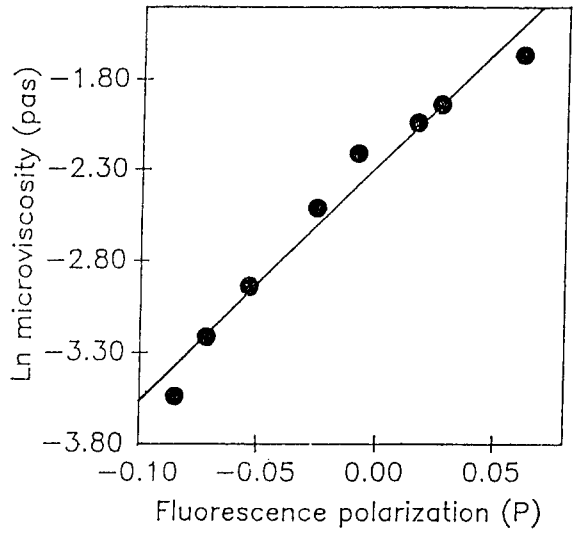

Fig. 8. Calibration curve of microviscosity on the fluorescence polarization (P) obtained from white mineral oil

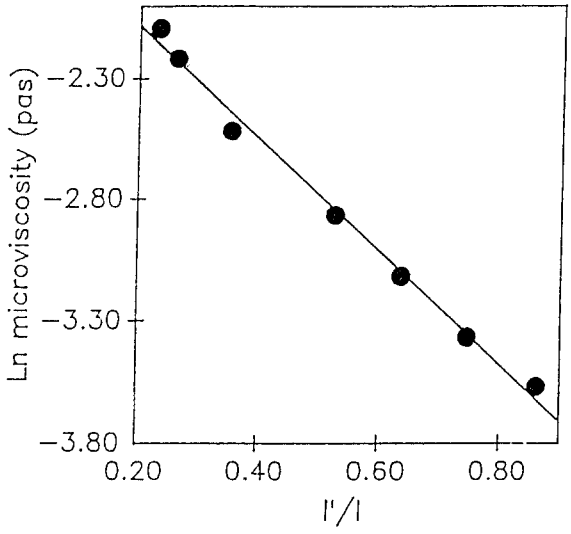

Fig. 9. Calibration curve of microviscosity on the excimer to monomer fluorescence intensity ratio (I'/I) obtained from white mineral oil

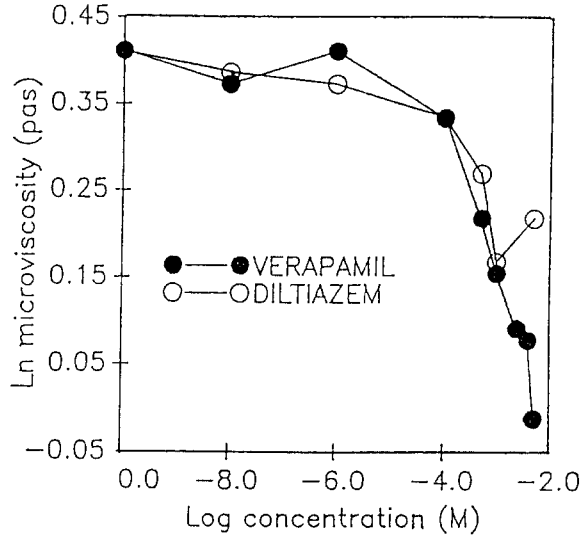

Fig. 10. Effects of verapamil and diltiazem on the microviscosity of the inner layer of erythrocyte ghost membranes at 20°C. Microviscosities were calculated from the calbration curve of fluorescence polarization.

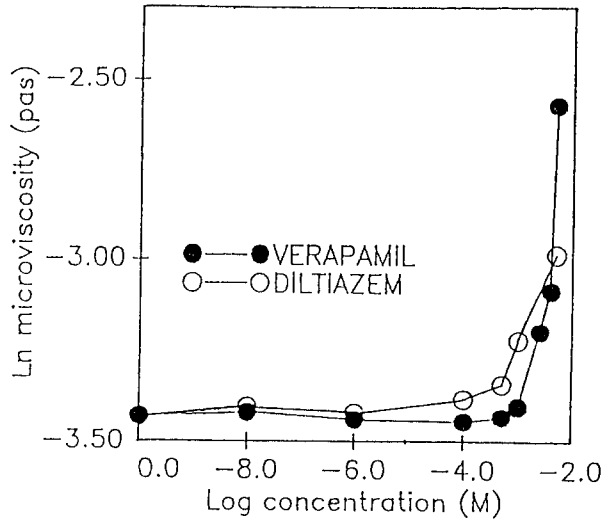

Fig. 11. Effects of verapamil and diltiazem on the microviscosity of the outer layer of erythrocyte ghost membranes at 20°C. Microviscosities were calculated from the calibration curve of excimer to monomer fluorescence intensity ratio.

ism for the interactions of various drugs with erythrocytes. It is proposed that such shape change may be due to an expansion of the lipid bilayer of biomembranes caused by a drug which selectively enters one of the two leaflets of the lipid bilayer. Tamura *et al* (1984) report that a similar type of shape change of human erythrocytes can also be produced by modifying only the outer half of the membrane lipd bilayer under nonhemolytic conditions with exogenously added phospholipase and these shape changes induced by enzyme treatments may also be due to an asymmetric expansion or shrinkage of the outer leaflet of the lipid bilayer relative to the inner one. Comparing the lipid fluidity of the outer and inner leaflets in normal erythrocytes treated experimentally to alter membrane cholesterol content, Flamn and Schachter (1982) have demonstrated that hemileaflet fluidity can be altered selectively.

The object of this study is primarily to measure the fluidity changes of ghost membranes by CAB's since Cogan *et al.* (1981) and Schachter *et al* (1982) have established that the lipid fluidity of the outer layer is greater than that of the inner leaflet of human erythrocytes. However, as confirmed from Fig. 10 and 11, it is a subject of interest that the selective changes of lipid fluidities of the outer and the inner leaflet can be occurred with progressive addition of verapamil

and diltiazem. It is found in this experiment that a decrease of hemolysis at low concentration of CAB's, as previously postulated by others (Peck and Lefer, 1981; Abe, *et al.*,; 1991) is consistant with an increase of the fluidity of inner layer . Therefore, a protective effect of CAB's against hemolysis of erythrocytes by its low concentrations is expected because the drug causes an expansion of the surface area of the cell membrane without a significant increase in the cell volume, thus large volume influx and it takes longer time to lyse.

CONCLUSION

In order to study the mechanism of the protective effect of CAB's on biomembranes, the fluidity of erythrocyte ghost membranes was determined with fluorescence probes, P and I'/I values of ghost membranes were evaluated into microviscosity comparing with the values of white mineral oil treated by the same manner as ghost membranes, and the following results were obtained;

1. Diltizem and nicardipine showed the inhibition effect against hypotonic hemolysis of erythrocytes at the concentration range of 10^{-6} to 10^{-5} M, while verapamil did at the concentration over 10^{-6} M.

2. The fluidity of the inner layer of ghost membranes was increased with an increase of the concentrations of verapamil and diltiazem from 10^{-8} M to 10^{-3} M, but any changes was not occurred in the fluidity of the outer layer.

3. In the case of nicardipine, it was difficult to get the reliable information, because of its low solubility in pH 7.4 phosphate buffer solution and its quenching effect with fluorescence probes used in this experiment.

4. It was considered that the protective effect of CAB's on erythrocyte ghost membranes was due to the expansion of the surface area relative to the volume of ghosts by their intercalation into the bilayer.

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