Effects of Diltiazem on the Physicochemical Properties of Rat Erythrocyte and Liposome Membrane: Comparison with Pentoxifylline and Propranolol

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Abstract—In vitro effects of the coronary vasodilator diltiazem on rat erythrocytes and liposomes were studied in comparison with propranolol and pentoxifylline. Diltiazem improved the deformability of rat erythrocytes, reduced the viscosity of rat erythrocyte suspensions, and protected the erythrocyte against hypotonic hemolysis at concentrations above $10^{-4}$ M, $10^{-5}$ M and $5 \times 10^{-7}$ M, respectively. Diltiazem at $5 \times 10^{-4}$ M also improved the impaired deformability of ATP-depleted erythrocytes, whereas it affected neither the adenine nucleotide level nor the phosphorylation of spectrin in the erythrocytes. Diltiazem enhanced the interaction of 1-anilino-naphthalene-8-sulfonate, a fluorescent probe, with erythrocyte ghosts at concentrations above $5 \times 10^{-6}$ M and, above $5 \times 10^{-5}$ M, inhibited the (Na$^+$+K$^+$)-ATPase activity of the erythrocyte ghosts. Diltiazem reduced the microviscosity of both erythrocyte ghosts and liposomes prepared from rat erythrocyte lipids. Diltiazem induced aggregation of the liposomes prepared from rat erythrocyte lipids, phosphatidylinositol, but it did not affect the liposomes prepared from a mixture of phosphatidylethanolamine and phosphatidylcholine (1:1). L-Diltiazem, a stereo-isomer of diltiazem, exhibited equipotent effects, compared with diltiazem, on these parameters. Propranolol showed similar properties, but pentoxifylline shared none of the above properties, except that it improved the deformability of erythrocytes. From these results, it is suggested that diltiazem may affect the erythrocyte membrane by interacting with acidic phospholipids and thus reduce the microviscosity of the membrane, improve erythrocyte deformability and protect the erythrocyte against hypotonic hemolysis.

Diltiazem, a potent coronary vasodilator which acts as a calcium entry blocker (1), also inhibits platelet aggregation in vitro (2). This inhibitory action of diltiazem on platelets seems to operate, at least in part, through a mechanism other than calcium antagonism because L-diltiazem, a stereoisomer of diltiazem, showed almost an equally potent inhibitory effect on platelet aggregation in spite of its very weak calcium-blocking activity compared with diltiazem (3). The finding that diltiazem inhibited both ADP-induced primary and secondary aggregation in human platelets at relatively high concentrations (above $10^{-4}$ M) (2) suggests that diltiazem, like many other membrane-active drugs such as chlorpromazine and propranolol (4–7), may exert the inhibitory action through a modification of physicochemical properties of the platelet membrane. In order to test this possibility, we examined effects of diltiazem on physicochemical properties of the membranes of rat erythrocytes and liposomes, and the effects were compared with those of propranolol and pentoxifylline.

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The results showed that diltiazem, like other membrane-active drugs, improves erythrocyte deformability, reduces microviscosity of erythrocyte membranes and protects the erythrocyte against hypotonic hemolysis, presumably by interaction with membrane lipids, especially, acidic phospholipids.

Materials and Methods

Materials: 1-Anilinonaphthalene-8-sulfonate (ANS), 1,6-diphenyl 1,3,5-hexatriene (DPH) and propranolol (dl) HCl were purchased from Nakarai Chemicals, Ltd. (Kyoto); L-α-phosphatidylserine (98%-pure) from Tokyo Chemical Industry Co., Ltd. (Tokyo); L-α-phosphatidylcholine (98%-pure) and L-α-phosphatidylethanolamine (98%-pure) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); L-α-phosphatidylethanolamine (98%-pure) from Green Cross Corporation (Osaka); Carrier-free [32P] phosphoric acid from New England Nuclear Corporation (Boston, U.S.A.). Diltiazem and /-diltiazem were synthesized at the Organic Chemistry Research Laboratory of our company (8). Pentoxifylline was extracted from Trental® (Hoechst Japan Co., Ltd.) and recrystalized at the Research Laboratory of Applied Biochemistry of our company.

Erythrocyte suspensions: Blood was withdrawn from the abdominal aorta of rats (male, Sprague-Dawley strain, 200–230 g) under ether anesthesia, immediately treated with heparin (20 units/ml blood), and then centrifuged at 500×g for 5 min. The resulting supernatant plasma (platelet rich plasma) and buffy coat were taken out by pipette and further centrifuged (1,000×g, 10 min) to give platelet poor plasma. The remaining bottom layer of erythrocytes was suspended in the platelet poor plasma to give a final hematocrit of 40% unless otherwise stated.

Erythrocyte ghosts: Erythrocyte ghosts were prepared from rat blood by the procedure of Dodge et al. (9), except that 10 mM Tris-HCl buffer (pH 7.4) was used in the hemolysis step.

ATP-depleted erythrocytes: A rat erythrocyte suspension was incubated with 16 mM NaF for 4 hr at 37°C. The erythrocytes were separated by centrifugation and washed four times with physiological saline. The ATP content in the NaF-treated erythrocytes was shown to be 3.69 μmoles as compared to 312.7 μmoles per 100 g hemoglobin in the untreated erythrocytes.

Liposomes: Liposomes were prepared from the lipid fraction extracted from rat erythrocyte ghosts or individual phospholipids by the method of Haxby et al. (10). Briefly, the lipid fraction from the erythrocyte ghosts (20 mg), phosphatidylserine (20 mg), phosphatidylinositol (20 mg) or a mixture of phosphatidylethanolamine (10 mg) and phosphatidylcholine (10 mg) was dispersed in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of 1 mg/ml and sonicated with an Ultra Sonic Disruptor (Model UR-200P, Tomy Seiko Co., Ltd., Tokyo) for 30 min at 20°C. The mixture was centrifuged (20,000×g, 1 hr), and the clear supernatant was used as liposome preparations.

Erythrocyte deformability: Erythrocyte deformability was measured by the filtration technique according to the method of Reid et al. (11). One ml of erythrocyte suspension (40%) was mixed with 1 ml of test compound solution in 2.69% NaCl and incubated for 1 min or 2.5 hr at 37°C. Then, 1 ml of the incubation mixture was filtered through a Nucleopore membrane filter (pore diameter: 5 μm, Nucleopore Corp., U.S.A.) under a negative pressure of 20 cm of water at room temperature, and the filtration time (sec/1 ml) was measured.

Viscosity of erythrocyte suspension: A 0.4 ml aliquot of erythrocyte suspension (80%) was mixed with 0.1 ml of test compound solution in 0.9% saline and incubated for 1 min or 2.5 hr at 37°C, and the viscosity at a shear rate of 37.5 S⁻¹ was measured with a cone-plate viscometer (48 cone, model E, Tokyo Keiki Co., Ltd., Tokyo).

Osmotic hemolysis: Half ml of erythrocyte suspension (40%) was mixed with 0.5 ml of test compound solution in 0.2% NaCl, incubated for 1 hr at 37°C and then centrifuged at 1,000×g for 10 min. The amount of hemoglobin released into the supernatant was measured by the CN-methemoglobin method (12) using a Shinotest Kit (Shinotest Laboratory Co., Ltd., Tokyo). The extent of hemolysis was expressed as percent of the
total hemolysis in distilled water.

Measurement of adenine nucleotides (ATP, ADP, AMP): Erythrocyte suspensions were incubated with a test compound for 2.5 hr at 37°C under the conditions for measurement of erythrocyte deformability and centrifuged (1,000 x g, 10 min). The erythrocytes were washed four times with physiological saline. The contents of adenine nucleotides in the erythrocytes were determined spectrophotometrically with a Hitachi spectrophotometer (Model 200-20, Hitachi Co., Ltd., Tokyo), employing the Boehringer (Mannheim, Germany) assay kits by the method described by Stefanovich (13).

Phosphorylation of spectrin: Incubation for phosphorylation of the erythrocyte spectrin was carried out according to the procedure reported by Nelson et al. (14). Four ml of rat erythrocyte suspension (40% in saline) were mixed with 4 ml of the incubation buffer (14) containing 5 mCi of carrier-free 32P-phosphoric acid and incubated for 17 hr at 37°C. The 32P-loaded erythrocytes were separated from the medium by centrifugation (500 x g, 10 min), followed by washing four times with 4 ml each of physiological saline and resuspended with saline to the initial volume. A portion of the washed 32P-loaded erythrocytes (2 ml) was further incubated with diltiazem (final conc. 5 x 10^-4 M, dissolved in 0.2 ml of the buffer) for 30 sec or 60 min at 37°C, and then lysed with distilled water to give a suspension of erythrocyte ghosts. Three aliquots (0.3 ml each) of the ghost suspension (200 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis according to the procedure of Fairbanks et al. (15). After staining with Coomassie Brilliant Blue, the bands of spectrin (band 1 + 2) were cut out, and their radioactivities were measured by the Cerenkov radiation method (16) with an Aloka Liquid Scintillation Spectrometer LSC-653 (Aloka Co., Ltd., Tokyo).

Interaction of the fluorescent probe with erythrocyte ghosts: Interaction of 1-anilino-naphthalene-8-sulfonate (ANS) with rat erythrocyte ghosts was studied by the method of Rubalcava et al. (17). A mixture of 3 ml ghost suspension in water (2.7 mg protein/ml) and 0.5 ml test compound solution in 10 mM Tris-HCl buffer (pH 7.4) was incubated for 5 min at 37°C. To this mixture was added 0.5 ml of ANS dissolved in the Tris-HCl buffer to give a final concentration of 2.7 x 10^-5 M. After standing for 1 hr at 37°C, the intensity of fluorescence was measured with a Hitachi Fluorescence Spectrophotometer MPF-2A (Hitachi Co., Ltd., Tokyo; excitation wave length, 350 nm; emission wave length, 470 nm).

Measurement of [Na^+ + K^+]-ATPase activity: Rat erythrocyte ghosts (10 mg protein/ml) were incubated with a test compound for 20 min at 37°C according to the procedure of Post and Sen (18), and released inorganic phosphate was determined by the method of Takahashi (19).

Measurement of microviscosity: Membrane microviscosity of rat erythrocyte ghosts and liposomes was measured by the procedure of Shinitzky et al. (20). One volume of the suspension of erythrocyte ghosts (800 μg protein/ml) or liposomes prepared from erythrocyte lipids (1 mg lipids/ml) was incubated with the same volume of 2 x 10^-6 M 1,6-diphenyl 1,3,5-hexatriene (DPH) dispersed in 10 mM Tris-HCl buffer (pH 7.4) for 1 hr at 37°C, and then the DPH-labeled ghosts or liposomes were washed once with 10 mM Tris-HCl buffer (pH 7.4) and resuspended with the buffer to the original volume. Two ml of the washed DPH-labeled ghosts or liposomes were mixed with 2 ml of test compound solution in Tris-HCl buffer and incubated for 1 hr at 37°C. Fluorescence polarization of the incubation mixture was measured with a Hitachi Fluorescence Spectrophotometer MPF-2A equipped with polarizers and a thermostatic cell holder (excitation wave length, 366 nm; emission wave length, 430 nm).

Measurement of liposome aggregation: Liposome aggregation was induced by addition of a test compound dissolved in 50 μl of 10 mM Tris-HCl buffer (pH 7.4) to 200 μl each of the liposome preparations and measured optically in a Sienco aggregometer (Model DP 247-E, Sienco Inc., Morrison, CO, U.S.A.) connected to a recorder (Model EPR-10A, Toa Electronics Ltd., Tokyo) under continuous agitation at 37°C.

Statistics: Most of the results were ex-
pressed as the mean±S.E.M. Student's t-test was used for statistical analysis.

**Results**

**Effects on erythrocyte deformability, blood viscosity and hypotonic hemolysis:** High osmolality is known to reduce erythrocyte deformability and thus impair its filterability (21). While 1 ml of the 20% rat erythrocyte suspension at physiological osmolarity was filtered almost instantaneously, the filtration speed slowed down to about 3 min per ml under the present hyperosmolar (1.35% NaCl) conditions. Incubation of the erythrocytes with diltiazem for 1 min partially prevented this reduction in erythrocyte deformability under the hyperosmolar conditions, as shown by the significant and concentration-dependent improvement of erythrocyte filterability in the presence of $10^{-4}$ M or higher concentrations of diltiazem (Fig. 1). Propranolol, known to affect the erythrocyte membrane structure probably by interacting with phospholipids (22), also improved the filterability to a similar extent in the same concentration range. Pentoxifylline, also known to improve the erythrocyte deformability (23), showed a similar, but less potent, effect compared to the above two drugs. Furthermore, /-diltiazem, one of the four optical isomers of diltiazem and only a weak calcium antagonist, was equipotent to diltiazem in this respect (Fig. 1). These improving effects on the erythrocyte

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**Fig. 1.** Effects of diltiazem, /-diltiazem, pentoxifylline and propranolol on rat erythrocyte deformability. One ml of erythrocyte suspension (40%) was mixed with 1 ml of test compound solution in 2.69% NaCl and incubated for 1 min (open) or 150 min (hatched) at 37°C before filtration. Erythrocyte filtration time was determined as described in the text. Each bar represents the mean±S.E. of 4 determinations. Statistical significance of the difference from the control: *P<0.05, **P<0.01. Dil, Diltiazem; Dil (/), /-Diltiazem; PE, Pentoxifylline; Prop, Propranolol.

**Fig. 2.** Effects of diltiazem, pentoxifylline and propranolol on the filterability of ATP-depleted erythrocytes. One ml of ATP-depleted erythrocyte suspension (40%, treated with 16 mM NaF for 4 hr at 37°C) was mixed with 1 ml of test compound solution in 2.69% NaCl and incubated for 1 min at 37°C before filtration. Erythrocyte filtration time was determined as described in the text. Each bar represents the mean±S.E. of 5 determinations. *P<0.05, **P<0.01.
filterability were unaltered after incubation for 2.5 hr.

Since erythrocyte deformability has been shown to be related to the ATP content of erythrocytes (24), we examined effects of diltiazem and other drugs on ATP-depleted erythrocytes. The filtration time of the ATP-depleted rat erythrocytes was significantly prolonged as shown in Fig. 2. Diltiazem and propranolol at $5 \times 10^{-4}$ M again improved the erythrocyte filterability as effectively as in the normal erythrocytes. However, pentoxifylline, which has been reported to exert this effect through elevation of the ATP contents (25), hardly showed any effect (Fig. 2). Diltiazem ($5 \times 10^{-4}$ M) did not influence the cellular levels of ATP, ADP and AMP ($297 \pm 8, 140 \pm 2$ and $24 \pm 1 \mu$moles per 100 g hemoglobin, respectively, in the presence of diltiazem versus $295 \pm 5, 137 \pm 2$ and $24 \pm 1 \mu$moles per 100 g hemoglobin, respectively, in its absence) of the rat erythrocytes after 2.5 hr of incubation. Whether the ATP level is involved in the effect of diltiazem on erythrocyte deformability has not been settled, because under the present conditions the adenine nucleotide levels of rat erythrocytes were not affected by pentoxifylline either (The ATP, ADP and AMP levels in the presence of $5 \times 10^{-4}$ M pentoxifylline were $295 \pm 7, 138 \pm 2$ and $25 \pm 1 \mu$moles per 100 g hemoglobin, respectively.).

Erythrocyte deformability is inversely related to blood viscosity (26). Diltiazem and propranolol reduced the viscosity of erythrocyte suspensions at concentrations above $10^{-5}$ M, whereas pentoxifylline did not (Fig. 3).

Propranolol and many other drugs which alter the physical properties of the cell membrane protect erythrocytes against hypotonic hemolysis (4). Diltiazem also protected

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**Fig. 3.** Effects of diltiazem, propranolol and pentoxifylline on the viscosity of rat erythrocyte suspensions. A 0.4 ml aliquot of erythrocyte suspension (80%) was mixed with 0.1 ml of test compound solution in 0.9% saline and incubated for 1 min at 37°C and the viscosity at a shear rate of 37.5 S$^{-1}$ was measured with a cone-plate viscometer (48° cone, model E, Tokyo Keiki Co., Ltd., Tokyo). Each point represents the mean of 3 determinations. (○—○) Diltiazem; (•—•) Propranolol; (×—×) Pentoxifylline.

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**Fig. 4.** Effects of diltiazem, propranolol and pentoxifylline on hypotonic hemolysis. Half ml of erythrocyte suspension (40%) was mixed with 0.5 ml of test compound solution in 0.2% NaCl, incubated for 1 hr at 37°C and then centrifuged at 1,000 g for 10 min. The amount of hemoglobin released into the supernatant from the erythrocytes was measured by the method described in the text. Extent of hemolysis was expressed as percent of total hemolysis in distilled water. Each point represents the mean±S.E. of 5 determinations. *P<0.05, **P<0.01, ***P<0.001. (○—○) Diltiazem; (△—△) / Diltiazem; (•—•) Propranolol; (×—×) Pentoxifylline.
the rat erythrocyte against hypotonic hemolysis in a concentration-dependent manner (Fig. 4) at concentrations above $5 \times 10^{-7}$ M. Propranolol showed a slightly more protective effect than diltiazem, whereas pentoxifylline had no such effect. Diltiazem was as active as diltiazem in this protective action.

Effect on the phosphorylation of spectrin:
The phosphorylation of spectrin is involved in the metabolism-dependent regulation of erythrocyte shape and deformability (27). The possibility that diltiazem might affect the phosphorylation of spectrin was examined. The counts of $^{32}$P incorporated into the pooled bands of spectrin after incubation for 30 sec or 60 min did not differ significantly whether the $^{32}$P-preloaded erythrocytes were incubated in the absence (1203±88 and 1109±149 cpm per 200 µg protein for the 30 sec and 60 min periods, respectively) or presence (1210±74 and 1047±30 cpm per 200 µg protein for the 30 sec and 60 min periods, respectively) of diltiazem.

Effects on microviscosity, ANS binding and [Na$^+$+K$^+$]-ATPase activity in rat erythrocyte ghosts: Propranolol has been reported to lower the fluorescence polarization (P) of DPH, a fluidity probe, embedded in erythrocyte membranes, suggesting that propranolol increases membrane fluidity (28). This effect of propranolol on membrane fluidity was confirmed with rat erythrocyte ghosts at concentrations over $5 \times 10^{-4}$ M (Fig. 5A). Diltiazem also showed the same property, although to a slightly less extent than propranolol, whereas pentoxifylline exhibited no such effect even at $5 \times 10^{-3}$ M (Fig. 5A). This effect of diltiazem on the fluidity of biological membranes was also observed when diltiazem was added to the liposome suspension prepared from rat erythrocyte lipids (Fig. 5B), suggesting that it is the lipids that react with diltiazem, resulting in various alterations of the physical properties of the erythrocyte membrane.

ANS is known to fluoresce strongly when

Fig. 5. Effects of diltiazem, propranolol and pentoxifylline on the fluorescence polarization (P) of DPH in rat erythrocyte ghosts and liposomes. One volume of the suspension of erythrocyte ghosts (800 µg protein/ml) (A) or liposomes prepared from erythrocyte lipids (1 mg lipids/ml) (B) was incubated with the same volume of $2 \times 10^{-6}$ M 1,6-diphenyl 1,3,5-hexatriene (DPH) dispersed in 10 mM Tris-HCl buffer (pH 7.4) for 1 hr at 37°C, and then the DPH-labeled ghost or liposomes were washed once with 10 mM Tris-HCl buffer (pH 7.4) and resuspended with the buffer to the original volume. Two ml of the washed DPH-labeled ghosts (0.8 mg protein/ml) or liposomes (1 mg lipid/ml) were mixed with 2 ml of test compound solution in Tris-HCl buffer and incubated for 1 hr at 37°C. Fluorescence polarization of the incubation mixture was measured as described in the text. Each point represents the mean±S.E. of 4 determinations. *P<0.05, **P<0.01, ***P<0.001. (○—○) Diltiazem; (●—●) Propranolol; (×—×) Pentoxifylline.
bound to hydrophobic regions of the erythrocyte ghost membrane (17). Figure 6 shows that diltiazem at concentrations above $5 \times 10^{-6}$ M increased the intensity of ANS fluorescence in a rat erythrocyte ghost suspension, suggesting a greater exposure of the hydrophobic regions to ANS. A similar effect was observed with propranolol, but not with pentoxifylline, even at $5 \times 10^{-4}$ M.

It is likely that drugs which alter the physical properties of biological membranes would affect the activities of membrane-bound enzymes. Diltiazem inhibited the $[\text{Na}^+ + \text{K}^+]$-ATPase activity of rat erythrocyte ghosts at concentrations above $5 \times 10^{-5}$ M, whereas pentoxifylline was without effect even at $5 \times 10^{-4}$ M (Fig. 7).

**Effects on aggregation of liposomes:** The $[\text{Na}^+ + \text{K}^+]$-ATPase of the erythrocyte membrane strongly depends on the presence of phosphatidylinerse (29), and propranolol has been reported to interact preferentially with acidic phospholipids (30). Therefore, physicochemical effects of diltiazem on liposomes of various compositions were examined by observing drug-induced aggregation of the liposomes as an interaction index (Fig. 8). Diltiazem induced aggregation as indicated by increments of the optical density of the liposome suspensions prepared from erythrocyte lipids or acidic phospholipids, but it did not affect the liposomes prepared from a mixture of phosphatidylethanolamine and phosphatidylcholine. Propranolol and $\beta$-diltiazem exhibited the same property as above, but pentoxifylline did not induce aggregation in any of the liposome preparations.

**Discussion**

The improvement of erythrocyte filterability and reduction in viscosity of the erythrocyte suspension by diltiazem (Figs. 1 and 3) are considered to reflect an increase in erythrocyte deformability. Since this effect of diltiazem on erythrocyte filterability was virtually instantaneous and time-independent (no difference at 1 min or 2.5 hr after ad-
Fig. 8. Effects of diltiazem, propranolol and pentoxifylline on the liposome aggregation. Liposome aggregation was induced by addition of test compound dissolved in 50 μl of 10 mM Tris-HCl buffer (pH 7.4) to 200 μl each of the liposome preparations and increases in optical density (an arbitrary but uniform sensitivity throughout the experiment) were measured in a Sienco aggregometer (Model DP 247-E, Sienco Inc., Morrison, CO, U.S.A.) connected to a recorder (Model EPR-10A, Toda Electronics Ltd., Tokyo) under continuous agitation at 37°C.

Propranolol was confirmed in the present study. Both /- and d-isomers of propranolol have the membrane-stabilizing action, but only the /-isomer has the β-adrenolytic property. As has been demonstrated with propranolol (22), diltiazem also caused configurational changes in the structure of erythrocyte membranes so as to allow the fluorescent probe ANS to fluoresce as a result of interaction with hydrophobic regions (Fig. 6) and depressed the activity of the membrane-bound \( [\text{Na}^+ + \text{K}^+] \)-dependent ATPase (Fig. 7), which is known to require the presence of acidic phospholipids (29). Preferential interaction of propranolol (30) and other cationic membrane-active drugs (32, 33) with acidic phospholipids has been established. Therefore, it is not surprising that both propranolol and diltiazem induced aggregation of the liposomes of acidic phospholipids, but not of neutral phospholipids (Fig. 8). These drugs probably interact with negatively charged functions of membrane phospholipids, reduce the electrostatic repulsive force between the liposomes, and allow them to aggregate. Thus, diltiazem is one of those non-stereoselective, membrane-active drugs which influence rheological properties of the red cell by interacting with acidic phospholipids such as phosphatidylserine in the cell membrane.

The platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34). Propranolol and many other membrane-active drugs inhibit the platelet primary aggregation (aggregation without the release reaction induced by ADP) has been interpreted as the platelet-to-platelet contact resulting from a membrane alteration (34).
both primary and secondary aggregation at concentrations (\( \sim 10^{-4} \) M) where the membrane effects on erythrocytes are observed (5-7). This effect is unrelated to a \( \beta \)-adrenergic blockade since the \( d \)-isomer, which lacks \( \beta \)-blocking activity, is as "membrane-active" as the \( \beta \)-adrenoletic \( l \)-isomer (5), and practolol, which possesses \( \beta \)-adrenoletic activity but lacks the membrane activity, does not inhibit platelet function (5). The same holds true for the calcium entry blocker diltiazem: i.e., diltiazem inhibits platelet aggregation (2), and \( l \)-diltiazem, which is only a weak calcium antagonist but as "membrane-active" as diltiazem (d), is equipotent as a platelet aggregation inhibitor (3). Therefore, it is very likely that diltiazem inhibits platelet aggregation in vitro by direct action on the platelet membrane. However, whether the membrane effect of diltiazem is totally unrelated to its calcium blocking activity is uncertain. The local anesthetic amines and other well studied membrane-stabilizing drugs such as procaine, chlorpromazine, quinidine and propranolol compete with and displace the membrane-bound calcium and inhibit the transmembrane fluxes of calcium (see reference 4 for a review). The reported negative inotropic and coronary dilating properties of these compounds (35) may be ascribed to their effects on calcium movement. Then, is the potent calcium blockade by diltiazem merely a result of potentiation of the membrane effect shared by a wide spectrum of drugs? Is the membrane-stabilizing activity prerequisite to the potent calcium blocking activity of known calcium antagonists? These questions cannot be answered until we encounter a calcium antagonist possessing no appreciable membrane activity. The calcium blockers (nifedipine, verapamil, TMB-8) so far examined all inhibited platelet aggregation as did diltiazem and propranolol (36). The chemical structures of the calcium antagonists have no outstanding common features except that all possess a bulky hydrophobic mass with a secondary or tertiary nitrogen in the periphery, which is also a common characteristic of many other membrane-active drugs. The lack of stereospecificity in the membrane action of these drugs may be indicative of an interaction with membrane lipids rather than membrane proteins.

Pentoxifylline, which is one of the methylxanthine-type cyclic AMP phosphodiesterase inhibitors and clinically used as a cerebral circulation-"improving" agent by virtue of its improving effect on erythrocyte deformability, is unique in that it improves only erythrocyte filterability and affects none of the other membrane parameters studied. This effect of pentoxifylline on erythrocyte filterability was no longer observed if the erythrocyte was depleted of ATP, suggesting that its mechanism of action is different from that of other membrane-active agents. There is a view that pentoxifylline increases the erythrocyte filterability by elevating cellular levels of ATP in the erythrocyte both in vivo and in vitro (13, 25). However, the results of the present study do not support this view.

Clinical significance of the membrane effects of diltiazem could not be assessed at present since the maximal plasma level of diltiazem attainable after a therapeutic dose (oral administration of 90 mg) is in the range of \( 2 \times 10^{-7} \) M (37), much lower than the concentrations that influence erythrocyte deformability, but high enough to inhibit hypotonic hemolysis. However, it should be noted that most of the known metabolites of diltiazem possess anti-aggregatory potencies greater than that of diltiazem (3). Since the effects of diltiazem on platelets and erythrocytes are in favor of prevention of thrombosis and facilitation of capillary blood flow, one may have to take this property into consideration in evaluating its therapeutic usefulness, especially when additive effects can be expected in the presence of its metabolites and other membrane-active drugs. Conversely, care should be taken not to always interpret in vitro effects of diltiazem as results of specific calcium blockade alone, especially when its concentration is above \( 10^{-6} \) M.

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