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Effects of Alcohols on ADP-Induced Aggregation and Membrane Fluidity of Gel-Filtered Bovine Blood Platelets

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Summary. The effects of four alcohols—*n*-propyl, *n*-butyl, *n*-amyl and *n*-hexyl alcohol—on the ADP-induced aggregation of gel-filtered bovine platelets were examined. All four alcohols inhibited the aggregation, the order of their effects being *n*-propyl < n-butyl < *n*-amyl < *n*-hexyl. Comparison of the inhibitory effects of the alcohols with their physico-chemical properties showed that their degrees of inhibition depended on their hydrophobicities. Moreover, it was suggested that their interaction with the lipid layer of the membrane was important for the inhibition. Studies on the effects of alcohols on the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene-labeled platelets showed that the membrane fluidity of the platelets increased in the same concentration range in which aggregation inhibition was observed. Since the alcohols inhibited aggregation without affecting Ca^{2+} mobilization in the platelets, as revealed in this study, it was concluded that inhibition of platelet aggregation was due to perturbation of membrane lipids by the alcohols. This hypothesis is supported by several recent studies on the effects of cholesterol and cations, which suggest that a relatively rigid membrane favors platelet aggregation.

Key Words alcohols · platelet aggregation · fluorescence polarization · membrane fluidity · structure-activity relationship

Introduction

A variety of pharmacologic agents have been employed to modify platelet aggregation activity. For example, compounds such as aspirin and indomethacin have been shown to act by inhibiting platelet cyclo-oxygenase (Cerletti, Livio & de Gaetano, 1981), an enzyme which catalyses the conversion of arachidonic acid to the cyclic endoperoxide (Moncada & Vane, 1979).

Platelet aggregation *in vitro* is also known to be inhibited by many amphiphilic substances such as fatty acids (MacIntyre, Hoover, Karnovsky & Salzman, 1980; Kanaho & Fujii, 1982; Srivastava & Awasthi, 1983), although the inhibitory mechanism is unknown. Alcohols are amphiphilic substances, but so far only ethyl alcohol has been shown to block platelet aggregation *in vitro* and *in vivo* (Haut & Cowan, 1974; Fenn & Littleton, 1982). Since alcohols have been found to increase the fluidity of other biological membranes, such as erythrocyte membranes (Chin & Goldstein, 1977), membrane perturbation as well as modification of membrane-bound phospholipase is postulated as a mechanism of inhibition by ethyl alcohol of aggregation (Fenn & Littleton, 1982).

In this work we examined the effects of four alcohols—*n*-propyl, *n*-butyl, *n*-amyl and *n*-hexyl alcohol—on the aggregation of gel-filtered bovine blood platelets and the relation between the inhibitory capacities of alcohols and their physicochemical properties. We examined the mechanism of inhibition by alcohols by comparing their inhibitory effects with their effects on the membrane fluidity of the platelets as determined by measuring fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene(DPH)-labeled platelets.

Materials and Methods

Materials

DPH, chlortetracycline (CTC) hydrochloride, bovine serum albumin (essentially fatty acid free), and bovine fibrinogen were purchased from Sigma Chemical Co. (St. Louis, Mo.). ADP was from Oriental Yeast Co. (Tokyo, Japan), and alcohols and other chemicals were from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Plasma-Free Suspension of Bovine Platelets

Fresh bovine (holstein) blood was collected in ACD coagulant solution (2.2% sodium citrate, 0.8% citric acid, and 2.2% dextrose), and centrifuged at 150 × *g* for 10 min at 20°C. The volume ratio of blood to ACD was about 9 : 1. Platelet-rich plasma (PRP) was separated and applied to a Sepharose 2B column equili-

brated with Ca^{2+}-free tyrode solution to obtain a plasma-free suspension of platelets (Tangen, Berman & Marfey, 1971). Platelet suspensions were diluted with Ca^{2+}-free tyrode solution to a final platelet concentration of about 1.8×10^5 (μl^{-1}).

Measurement of Aggregation

A solution of 1 mg · ml^{-1} albumin and 1 mg · ml^{-1} fibrinogen containing an equal volume of Ca^{2+}-free tyrode solution was added to the platelet suspension obtained as described above. Then $CaCl_2$ was added at a final concentration of 0.2 mM. After addition of various concentrations of alcohols, ADP was added to the suspension at 37°C at a final concentration of 2 μM, and the optical density change at 600 nm was recorded in a spectrophotometer UV-180 (Schimadzu Seisakusho Co., Kyoto, Japan) equipped with a stirrer and thermostat. The rate of aggregation was measured as the steepest tangential slope of the downward deflection of the records. The effects of alcohols on aggregation are expressed as aggregation rates with alcohols relative to those without alcohols, as described previously (Kitagawa, Endo, Kubo & Kametani, 1983).

Measurement of Fluorescence Polarization

Fluorescence polarization of platelets was measured in the platelet suspension described above. The fluorescence probe DPH was used to label platelets, as described by Kowalska and Cierniewski (1983). Platelets were incubated at a concentration of about 9×10^4 (μl^{-1}) with 1×10^{-6} M DPH for 40 min, and the DPH fluorescence in the platelets was measured in a spectrofluorometer 650-40 (Hitachi Seisakusho Co., Tokyo, Japan) at 37°C. The excitation and emission wavelengths were 360 and 428 nm, respectively. A polarized excitation beam was used in measurement of fluorescence intensities. Fluorescence polarization (F_p) was calculated according to the equation

$$F_p = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with a vertical polarizer and analyzer mounted vertically and horizontally, respectively. $G = I_{HV}/I_{HH}$ is the correction factor (Chen & Bowman, 1965).

Measurement of Ca^{2+} Mobilization

Fluorescence of CTC was employed as a probe to measure Ca^{2+} mobilization. CTC-labeled platelets were prepared as described previously (LeBreton, Dinerstein, Roth & Feinberg, 1976; Feinstein, 1980). CTC fluorescence in the same concentration of platelets as mentioned above was measured at 37°C in the spectrofluorometer described above. The excitation and emission wavelengths were 390 and 528 nm, respectively, which were almost the same as those for human platelets (LeBreton et al., 1976; Feinstein, 1980).

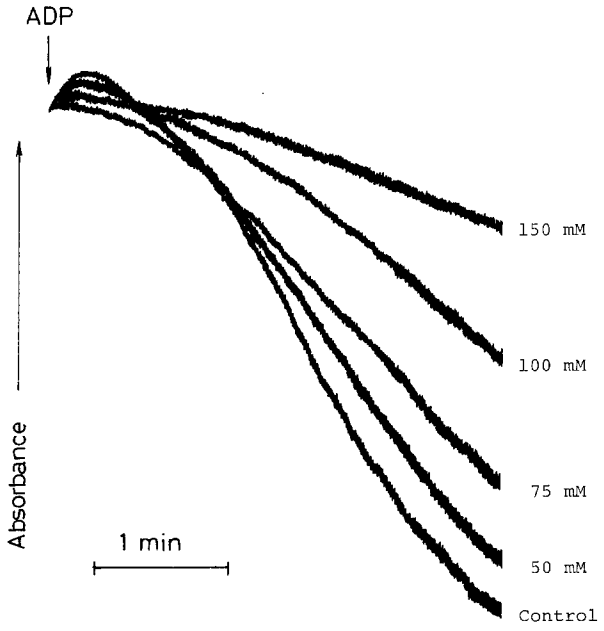

Fig. 1. Effect of *n*-butyl alcohol on 2 μM ADP-induced aggregation. Aggregation was examined in a solution containing final concentrations of 0.5 mg · ml^{-1} albumin, 0.5 mg · ml^{-1} fibrinogen, and 0.2 mM $CaCl_2$. The concentrations of *n*-butyl alcohol used are shown

Results

Inhibition of Platelet Aggregation by Alcohols

Addition of 2 μM ADP to a suspension of bovine gel-filtered platelets caused a decrease in the absorbance, indicating aggregation of the platelets. The aggregation was inhibited by the four alcohols tested, as shown for *n*-butyl alcohol in Fig. 1. Moreover, the shape change of the platelets induced by ADP, which is indicated by an initial increase in absorbance just after the addition of ADP, was also inhibited by the alcohols. The inhibitory effects of the alcohols on aggregation were in the order *n*-hexyl > *n*-amyl > *n*-butyl > *n*-propyl. Ethyl alcohol had no significant effect at concentrations of under 400 mM in our experimental conditions. These results indicate that the inhibition increased with the length of the hydrocarbon chain of the alcohols.

Relation of Inhibitory Effects to Physico-Chemical Properties of Alcohols

To establish the quantitative relation of the physicochemical properties of the alcohols with their inhibition of aggregation, we examined the correlation between their inhibitory effects and a hydrophobic parameter, the partition coefficient P, according to

the method introduced by Hansch (Hansch et al., 1963). This method has been applied in studies of various biological activities of alcohols and other organic compounds, such as hemolysis and membrane transport (Hansch & Glave, 1971; Kitagawa, Terada & Kametani, 1982), but not yet in studies on modification of platelet functions. The inhibitory effects of alcohols on aggregation were expressed as the reciprocals of their ID_{50} values (i.e., concentrations inducing 50% inhibition of aggregation). The logarithms of the $1/ID_{50}$ values of the alcohols and of the partition coefficients in an *n*-octyl alcohol/H_2O system P_{oct} are listed in Table 1.

The quantitative relation between the inhibitory effects and hydrophobic parameters is shown by Eq. (1).

$$\log(1/ID_{50}) = 1.00 \log P_{oct} - 2.82 (n = 4, r = 0.997). \quad (1)$$

Here, *n* is the number of compounds tested and *r* is the correlation coefficient. From the high correlation shown by this equation, it is concluded that the hydrophobic properties of alcohols determine their inhibitory effects on platelet aggregation. This finding suggests that the interaction of alcohols with the lipid bilayer of the membrane or with hydrophobic regions of proteins is important for the inhibition.

Table 1. Relation between partition coefficients, P_{oct}, of alcohols and their inhibitory effects on aggregation, $1/ID_{50}$ values

Alcohol	log P_{oct}[a]	$\log(1/ID_{50})$ (mM^{-1})
n-Propyl	0.34	−2.44 ± 0.04
n-Butyl	0.88	−1.99 ± 0.04
n-Amyl	1.40	−1.48 ± 0.04
n-Hexyl	2.03	−0.76 ± 0.05

[a] Values of log P_{oct} are cited from Leo, Hansch and Elkins (1971).

Membrane Fluidity Change by Alcohols

Next we examined the effects of alcohols on the membrane fluidity by measuring fluorescence polarization of DPH-labeled platelets. DPH has been used as a fluorescent indicator of changes in the fluidity of the membrane lipid bilayer. There have been many studies using this probe on the effects of various substances on the fluidity of the platelet membrane (Shattil & Cooper, 1976; Steiner, 1981; Kowalska & Cierniewski, 1983). As shown in Fig. 3, results using this probe showed that the alcohols decreased the fluorescence polarization, indicating that they increased membrane fluidity (Giraud, Claret, Bruckdorfer & Chailley, 1981; Fukuzawa, Chida, Tokumura & Tsukatani, 1981). The capacities of the alcohols to increase fluidity were also in the order *n*-hexyl > *n*-amyl > *n*-butyl > *n*-propyl. Comparison of the results in Figs. 2 and 3 shows that the fluidity increased in the same concentration range of each alcohol at which aggregation inhibition occurred, except that *n*-propyl alcohol induced fluidity change at a slightly lower concentration than that for inhibition of aggregation.

Effects of Alcohols on Ca^{2+} Mobilization

To determine the inhibitory mechanisms of alcohols, we next examined their effects of Ca^{2+} mobilization by measuring fluorescence of CTC-labeled platelets. On addition of ADP to bovine platelets,

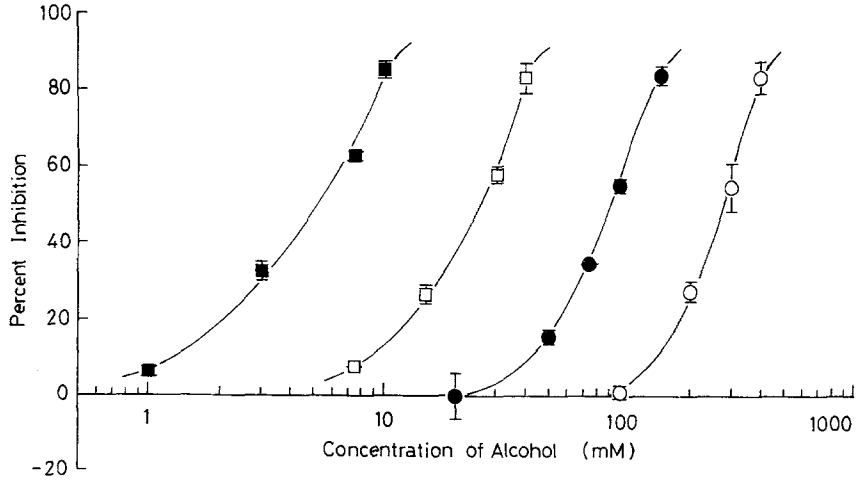

Fig. 2. Dose-response curves for inhibition of 2 μM ADP-induced aggregation by alcohols. The abscissa shows the concentration of alcohols on a logarithmic scale, and the ordinate the percent aggregation inhibition. Symbols: ○, *n*-propyl; ●, *n*-butyl; □, *n*-amyl; ■, *n*-hexyl alcohol. Data are mean values ± SD for three experiments

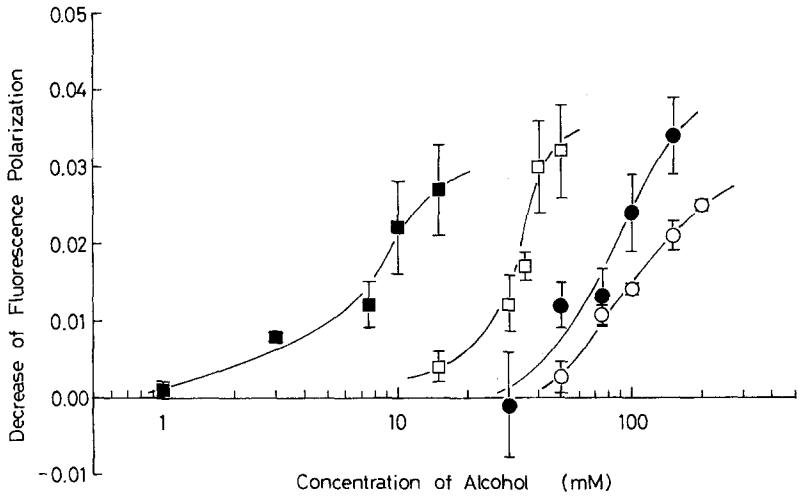

Fig. 3. Effects of alcohols on the fluorescence polarization of DPH-labeled platelets. The abscissa shows the concentration of alcohols on a logarithmic scale, and the ordinate the decrease of fluorescence polarization on addition of alcohols. Symbols: ○, *n*-propyl; ●, *n*-butyl; □, *n*-amyl; ■, *n*-hexyl alcohol. Data are mean values ± SD for three or four experiments. The control value of fluorescence polarization was 0.197 ± 0.021

the fluorescence intensity decreases, indicating transfer of Ca^{2+} from storage pools, such as the dense tubular system, to the cytosol (Owen & LeBreton, 1981). Several inhibitors of platelet aggregation have been shown by this fluorescence technique to inhibit this Ca^{2+} mobilization. These compounds include inhibitors of thromboxane A_2 synthesis, such as indomethacin, and reagents increasing the intracellular concentration of cAMP, such as prostaglandin I_2 (Owen & LeBretin, 1981). However, as shown in Table 2, addition of the alcohols at the concentrations that inhibited aggregation had no appreciable effect on Ca^{2+} mobilization induced by ADP.

Discussion

A variety of pharmaceutical agents have been found to inhibit platelet functions. Among these agents, nonsteroidal anti-inflammatory drugs, such as aspirin, have been shown to act by inhibiting platelet cyclo-oxygenase (Cerletti et al., 1981), and drugs such as prostaglandin I_2 by activating adenylate cyclase and increasing the intracellular concentration of cAMP (Gorman, Fitzpatrick & Miller, 1978). These agents seem to inhibit platelet functions by inhibiting Ca^{2+} mobilization directly or indirectly (LeBreton, Owen & Feinberg, 1982).

Fenn and Littleton (1982) suggested that ethyl alcohol might inhibit aggregation of human platelets by decreasing the activity of membrane-bound phospholipase, which is important for regulation of Ca^{2+} mobilization. However, this mechanism is improbable because we found that alcohols inhibited aggregation without affecting Ca^{2+} mobilization. The high correlation observed between the inhibitory effects and hydrophobic parameters of alcohols suggests that the interaction of alcohols with the lipid bilayer of the membrane is important for the inhibition, although their interaction with the hydrophobic region of proteins must also be considered.

Since measurement of fluorescence polarization indicated that the membrane fluidity increased in the same concentration range of each alcohol at which aggregation inhibition occurred, the inhibitory effects of alcohols seemed to be due to membrane perturbation. This hypothesis is supported by several recent studies on the effects of cholesterol and cations, suggesting that the relative fluidity of the plasma membrane may have functional significance (Shattil & Cooper, 1976; Sauerheber et al., 1980). Shattil and Cooper (1976) observed increased sensitivity of cholesterol-rich platelets to aggregating agents and its correlation with reduction of membrane fluidity. Moreover, Sauerheber et al. (1980) observed reduction of membrane fluidity of platelets on addition of Ca^{2+}, an ion required for platelet aggregation (Born & Cross, 1964). These results and the data presented here suggest that a relatively rigid membrane may favor platelet aggregation, although the reason for this is unknown.

As suggested by Sauerheber et al. (1980), the increased membrane fluidity may suppress platelet functions by modifying various membrane-associated functions. As proposed for other biological membranes such as erythrocyte membranes (Deuticke, 1977; Hirata & Axelrod, 1980), the membrane fluidity may affect the transport rates of ions and several enzymatic activities. Moreover, a change in membrane fluidity may affect the cytoskeletal network because of its interaction with the lipid bilayer (Noji, Takahashi & Kon, 1982). Since reorganization of cytoplasmic contractile and structure proteins is essential for platelet functions

Table 2. Effects of alcohols on ADP-induced CTC fluorescence change

Alcohol	Conc.(mM)	$\Delta F(\%)^a$
Control		5.57 ± 0.37
n-Propyl	200	5.14 ± 0.53
n-Butyl	100	5.85 ± 0.65
n-Amyl	30	6.12 ± 0.45
n-Hexyl	8	5.57 ± 0.34

[a] ΔF means the percentage decrease in the fluorescence in the initial 30 sec induced by 2 μM ADP. Values are means ± SD for three experiments.

(Jennings, Fox, Edwards & Phillips, 1981; Pribluda & Rotman, 1982), perturbation of the lipid bilayer by reagents such as alcohols may inhibit platelet aggregation by modifying this reorganization. The inhibition by the alcohols tested of ADP-induced shape change of platelets (Fig. 1) supports this idea.

Membrane fluidity has been found to be altered by dietary constituents and in various diseases due to modification of the phospholipid fatty acid composition and the cholesterol content (Kitajima & Shibata, 1975; Berlin, Matusik & Young, 1980). It is especially important to determine the relation between membrane fluidity and platelet functions for preventing arterial thrombosis. Further studies on this problem are in progress.

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