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Relationship of Alcohol-induced Changes in Mg^{2+}-ATPase Activity of Rabbit Intestinal Brush Border Membrane with Changes in Fluidity of Its Lipid Bilayer

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Abstract. We examined the effects of seven n-alkyl alcohols (from n-butyl to n-undecyl alcohol), isoamyl alcohol and benzyl alcohol on the activity of membrane enzyme Mg^{2+}-ATPase of the rabbit small intestinal brush border membrane. Their relationships with the changes in the fluidity of the membrane lipid bilayer were examined through studies on the fluorescence anisotropies of diphenylhexatriene (DPH) and its ionic derivatives. Good linear correlations were found both between the partition coefficients of the alcohols and their concentrations causing similar decreases in the activity of Mg^{2+}-ATPase and between their partition coefficients and the alcohol-induced changes in fluorescence anisotropies. Within the concentration range of the alcohols tested, including isoamyl alcohol and benzyl alcohol, the decreases in activity of the membrane enzyme Mg^{2+}-ATPase clearly corresponded with the decreases in fluorescence anisotropy of DPH, which is thought to be located within the hydrophobic core of the membrane. From these findings, one possible explanation is that inhibition of this enzyme by the alcohols is due to perturbation of the lipid bilayer of the brush border membrane.

Key words: Brush border membrane — Alcohol — Mg^{2+}-ATPase — Membrane fluidity — Fluorescence — Small intestine

Introduction

Membrane fluidity has been found to have important influences on the activities of membrane enzymes and the properties of membrane transport (Stubbs & Smith, 1984; Le Grimellec et al., 1992). Amphiphilic compounds such as alcohols are reported to modify these membrane functions by changing membrane fluidity (Garda & Brenner, 1984; Chin & Goldstein, 1985; Kitagawa et al., 1990; Le Grimellec et al., 1992). However, there are many points that have not been clarified. For example, quantitative relationships between the changes in fluidity induced by various amphiphilic compounds and the changes in membrane functions have not been clarified. Therefore, it is also unknown whether the relationships between them are the same or not for homologous series of compounds such as n-alkyl alcohols and other compounds with different chemical structures.

The effects of n-alkyl alcohols on the activity of Mg^{2+}-ATPase, one of the enzymes enriched in the brush border membrane, have been examined (Mitjavila, Lacombe & Carrera, 1976; Fernandez et al., 1984). Mitjavila et al. (1976) reported that eight n-alkyl alcohols, from methyl alcohol to n-octyl alcohol, inhibited the activity of this enzyme from the rat small intestine. The inhibition depended on the concentration of the alcohols and their alkyl chain lengths. However, Fernandez et al. (1984) in the same group, observed a decrease of the dose-dependency of the inhibitory effects of alcohols with an increase in their alkyl chain lengths. They did not find any correlation between the effects of the alcohols on membrane fluidity and their inhibitory effects on this enzyme.

In this study, we attempted to determine whether or not there is a correlation between the alcohol-induced changes in membrane fluidity and the changes in activities of membrane enzyme Mg^{2+}-ATPase. We examined the effects of seven n-alkyl alcohols on depth-dependent fluidity in the lipid bilayer and their relation to the inhibitory effects of the alcohols on Mg^{2+}-ATPase in rabbit small intestinal brush border membrane. We observed the fluorescence anisotropies of diphenylhexatriene

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(DPH),[1] a cationic trimethylammonium derivative (TMA-DPH) and an anionic propionic acid derivative (DPH-PA), located at different depths in the lipid bilayer (Kuhry et al., 1983; Trotter & Storch, 1989; Kitagawa et al., 1991). The effects of isoamyl alcohol and benzyl alcohol on the fluidity and the activity of the enzyme were also compared with those of seven n-alkyl alcohols. Whether there is a common relationship for all the alcohols tested between the changes in membrane fluidity and the changes in activities of this enzyme was also examined.

Materials and Methods

Materials

TMA-DPH and DPH-PA were purchased from Molecular Probe (Junction City, OR). DPH, Tris salt of ATP and ouabain were from Sigma (St. Louis, MO). Alcohols and other reagents were all from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Brush Border Membrane Vesicles

Brush border membrane vesicles were isolated from the rabbit small intestine by the $CaCl_2$ precipitation method of Kessler et al. (1978). The membrane vesicles obtained were suspended in experimental buffer (100 mM mannitol, 100 mM KCl and 10 mM HEPES-KOH (pH 7.5).

Determination of Mg^{2+}-ATPase Activity

Mg^{2+}-ATPase activity was measured as described by Fernandez et al. (1984). A sample of 0.1 ml vesicle suspension in experimental buffer (51.5 μg protein) was mixed with 0.5 ml containing (in mM): 2 ouabain, 15 $MgCl_2$, 240 NaCl, 40 KCl and 60 Tris-HCl (pH 7.4) and preincubated at 37°C for 15 min in the presence of various concentrations of alcohols. The concentration of membrane phospholipids in this vesicle suspension was about 36 μM. Then 0.2 ml of 25 mM Tris-ATP was added, and incubation at 37°C was continued for 15 min. The reaction was stopped by the addition of 0.25 ml of 2.5 M $HClO_4$ and proteins were removed by centrifugation. The amount of Pi liberated was determined by the Fiske-SubbaRow method (Fiske & SubbaRow, 1926).

Measurement of Fluorescence Anisotropy

The fluorescence anisotropies of DPH and its derivatives in the brush border membrane were measured as described previously (Kitagawa et al., 1991). A suspension of the membrane vesicles described above was mixed with the experimental buffer in which various concentrations of alcohols were dissolved by vigorous vortex mixing at 37°C after either direct addition of alcohols (n-butyl, n-amyl, n-hexyl, isoamyl and benzyl alcohols) or addition of methanol solution of alcohols (n-heptyl, n-octyl, n-decyl and n-undecyl alcohols). The suspension was then incubated with a final concentration of 0.5 μM TMA-DPH or DPH-PA at 37°C for 2 min or with 0.5 μM DPH for 4 min before measurements. The fluorescence intensities of these probes were measured at 37°C in an F-4010 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan) equipped with excitation and emission polarizers. The excitation and emission wavelengths used for DPH, TMA-DPH and DPH-PA were 363 and 428 nm, 365 and 428 nm and 366 and 430 nm, respectively. Steady-state fluorescence anisotropy was calculated as described previously (Kubina et al., 1987).

[1] *Abbreviations:* DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; DPH-PA, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid.

Table. Effects of alcohols on activity of Mg^{2+}-ATPase

Alcohol	Conc. (mM)	Relative Activity[a]
n-Butyl	50	90.4 ± 2.8
	100	83.5 ± 1.3
	200	63.5 ± 2.0
n-Amyl	10	94.9 ± 1.2
	25	87.7 ± 3.4
	50	68.7 ± 1.4
n-Hexyl	2.5	94.5 ± 2.5
	5.0	90.2 ± 2.1
	10	82.4 ± 2.1
	20	75.6 ± 2.0
n-Heptyl	1.0	93.4 ± 2.1
	2.5	87.6 ± 2.1
	5.0	76.3 ± 2.0
n-Octyl	0.4	95.3 ± 3.0
	1.0	91.4 ± 4.2
	2.0	79.3 ± 2.8
n-Decyl	0.05	92.8 ± 2.2
	0.1	89.1 ± 2.2
	0.2	81.1 ± 2.2
n-Undecyl	0.02	91.6 ± 1.8
	0.04	88.6 ± 3.7
	0.08	82.7 ± 2.4
Isoamyl	50	91.6 ± 1.4
	75	81.5 ± 0.8
	100	72.2 ± 1.3
Benzyl	20	90.8 ± 2.9
	40	86.4 ± 0.4
	100	66.9 ± 3.1

[a] Control activity (5.56 ± 0.33 mols Pi/15 min per mg protein) was defined as 100. Values are means ± SD for three experiments. Relative activities in the presence of the alcohols listed in this table were all significantly different from the control value at $P < 0.001$.

Results

Effects of Alcohols on Activities of Mg^{2+}-ATPase

We first examined the effects of seven n-alkyl alcohols (n-butyl, n-amyl, n-hexyl, n-heptyl, n-octyl, n-decyl and n-undecyl alcohols), isoamyl alcohol and benzyl alcohol on the activity of the brush border membrane enzyme Mg^{2+}-ATPase. As shown in the Table, all the alcohols including isoamyl alcohol and benzyl alcohol inhibited the activity of this enzyme in a dose-dependent manner.

The inhibitory effects of the alcohols increased with an increase in their alkyl chain length, consistent with the findings by Mitjavila et al. (1976). The concentrations of the alcohols that inhibited Mg^{2+}-ATPase were similar to those concentrations of the same alcohols that modified the transport of various solutes and enzymatic activities in other biological membranes (Gordon et al., 1980; Friedlander et al., 1987; Orme, Moronne & Macey, 1988; Schwichtenhövel, Deuticke & Haest, 1992). However, Mg^{2+}-ATPase was less sensitive to n-alkyl alcohols than some membrane transport proteins such as voltage-gated potassium channel in identified neurons from Helix aspersa (Winpenny, Elliot & Harper, 1992) and the choline transport system in erythrocytes (Devés & Krupka, 1990).

As shown in the Table, we examined the correlation between the hydrophobicities of the alcohols and their inhibitory potencies on the activity of the Mg^{2+}-ATPase. Their inhibitory potencies were expressed as reciprocals of their concentrations for inhibiting the activity of this enzyme by a certain percentage. The hydrophobicities of the alcohols were expressed as their partition coefficients between n-octyl alcohol and water, P_{oct} values, as described by Hansch & Dunn (1972). For example, as shown in Fig. 1, the logarithmic values of the reciprocals of their IC_{10} (Mg) values, which represent the concentrations at which alcohols induced a 10% decrease of this enzyme activity, increased linearly with the increase in logPoct, fitting the following equation:

$$\log(1/IC_{10}(Mg))\ (M^{-1}) = 0.897\ \log P_{oct} + 0.430$$

$$(n = 9,\ r = 0.994) \qquad (1)$$

As shown in Fig. 1, the effects of isoamyl alcohol and benzyl alcohol on Mg^{2+}-ATPase activity were also shown to be simply dependent on their partition coefficients.

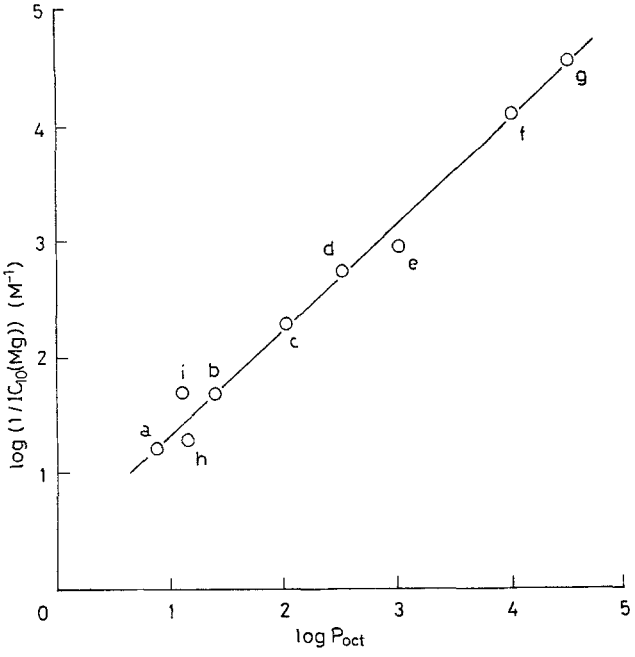

Fig. 1. Relationship between logarithmic values of partition coefficients, P_{oct} values, of alcohols and logarithmic values of the reciprocals of their IC_{10} (Mg) values that represent the concentrations at which alcohols induced 10% decrease in Mg^{2+}-ATPase activity: *a*, n-butyl; *b*, n-amyl; *c*, n-hexyl; *d*, n-heptyl; *e*, n-octyl; *f*, n-decyl; *g*, n-undecyl; *h*, isoamyl; *i*, benzyl. IC_{10} (Mg) values were obtained from the data shown in the Table. P_{oct} values are cited from Hansch & Dunn (1972).

Effects of Alcohols on the Fluidity of the Lipid Bilayer of the Brush Border Membrane and their Relation to Changes in Mg^{2+}-ATPase Activities

We next examined the effects of these alcohols on the fluorescence anisotropies of DPH, TMA-DPH and DPH-PA to determine the effects of these alcohols on membrane fluidity at different depths in the lipid bilayer of the brush border membrane. DPH is considered to be located within the hydrophobic core of the lipid bilayer and to provide information on the membrane fluidity in this region (Trotter & Storch, 1989). On the other hand, ionic derivatives seem to be anchored in proximity to the bilayer surface, and so provide information on the bilayer lipid environment rather near the surface (Kuhry et al., 1983; Trotter & Storch, 1989). Brush border membranes from mammalian small intestine have been shown to be more rigid than other plasma membranes through spin-labeling studies (Hauser et al., 1982). This seems to be reflected in the relatively large fluorescence anisotropies of these fluorescent probes shown in Fig. 2. In the absence of alcohols, the fluorescence anisotropy of TMA-DPH was larger than that of DPH-PA (Fig. 2). This difference seems to reflect the difference in the localizations of these probes in the membrane due to their difference of electronic charge, as in the platelet membranes we observed previously (Kitagawa et al., 1991). The cationic probe may be located in a more rigid environment. Since the fluorescence anisotropies of these ionic probes were almost the same in liposomes of extracted lipids from the brush border membrane (*data not shown*), this difference may reflect the asymmetrical distribution of the lipids in the brush border membrane (Lipka, Op den Kamp & Hauser, 1991).

As shown in Fig. 2 for n-hexyl alcohol, these alcohols decreased the fluorescence anisotropy of DPH at the concentrations that inhibited the activity of Mg^{2+}-ATPase. We next examined the correlation of the potencies of the effects of alcohols on the decrease in fluorescence anisotropy of DPH with the hydrophobicities of the alcohols. The potencies of alcohols in decreasing the fluorescence anisotropy of DPH were expressed as the reciprocals of their concentrations for decreasing the

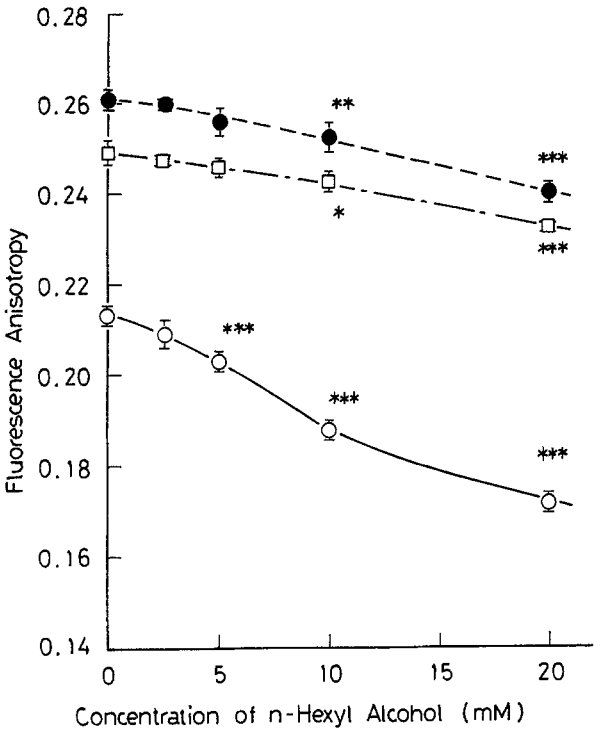

Fig. 2. Effects of n-hexyl alcohol on fluorescence anisotropies of DPH (○), TMA-DPH (●) and DPH-PA (□) in brush border membranes. Membrane vesicle suspensions were incubated with final concentrations of 0.5 μM TMA-DPH or DPH-PA at 37°C for 2 min or with 0.5 μM DPH for 4 min before measurements. Data are means ± SD for four experiments. The significance of differences between control values and those in the presence of n-hexyl alcohol were determined by Student's *t*-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

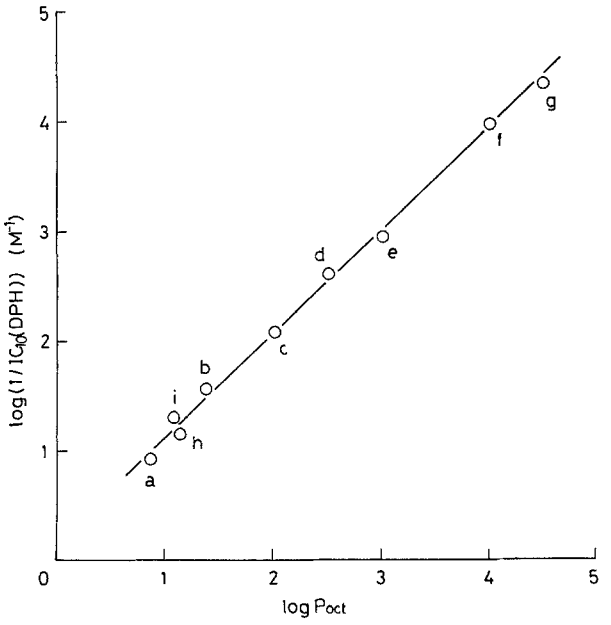

Fig. 3. Relationship between logarithmic values of partition coefficients, P_{oct} values, of alcohols and logarithmic values of the reciprocals of their IC_{10} (DPH) values, representing the concentrations at which alcohols induced 10% decrease in fluorescence anisotropy of DPH in brush border membranes: *a*, n-butyl; *b*, n-amyl; *c*, n-hexyl; *d*, n-heptyl; *e*, n-octyl; *f*, n-decyl; *g*, n-undecyl; *h*, isoamyl; *i*, benzyl. IC_{10} (DPH) values were obtained from the dose-dependent effects of the alcohols on the fluorescence anisotropy of DPH as shown in Fig. 2 for n-hexyl alcohol. P_{oct} values are cited from Hansch & Dunn (1972).

fluorescence anisotropy of DPH by a certain percentage. A good correlation between these two values was found for all the alcohols, including isoamyl alcohol and benzyl alcohol at the concentration range tested. For example, as shown in Fig. 3, the logarithmic values of the reciprocals of the IC_{10} (DPH) values of alcohols, which represent their concentrations for inducing a 10% decrease in fluorescence anisotropy of DPH, increased linearly with increase in log P_{oct}, fitting the following equation:

$$\log(1/IC_{10}(DPH))\ (M^{-1}) = 0.931\ \log P_{oct} + 0.177$$

$$(n = 9,\ r = 0.998) \qquad (2)$$

where n is the number of data, and r is the correlation coefficient. These findings are consistent with those on the correlation between the hydrophobicities of these alcohols and their inhibitory potencies on Mg^{2+}-ATPase shown in Fig. 1 and Eq. (1).

Similar correlations with the partition coefficients were also found for cationic TMA-DPH and anionic DPH-PA, but only at relatively low concentrations of the alcohols. For example, as shown in the following Eqs. (3) and (4),

$$\log(1/IC_5(TMA\text{-}DPH))\ (M^{-1}) = 0.967\ \log P_{oct} + 0.148$$

$$(n = 9,\ r = 0.996) \qquad (3)$$

$$\log(1/IC_5(DPH\text{-}PA))\ (M^{-1}) = 0.927\ \log P_{oct} + 0.143$$

$$(n = 9,\ r = 0.994) \qquad (4)$$

for the logarithmic values of the reciprocals of IC_5 (TMA-DPH) and IC_5 (DPH-PA) values, which represent the concentrations of the alcohols for inducing 5% decreases in fluorescence anisotropies of TMA-DPH and DPH-PA, respectively, similar linear correlations were observed as for DPH. However, significant correlations were not observed at the concentration range of the alcohols that induced more than 5% decreases in fluorescence anisotropies of these probes.

To examine the correlation between the effects of the alcohols on fluorescence anisotropy of DPH and their inhibition of Mg^{2+}-ATPase, we plotted the data as shown in Fig. 4. Clearly, the percentage decrease of Mg^{2+}-

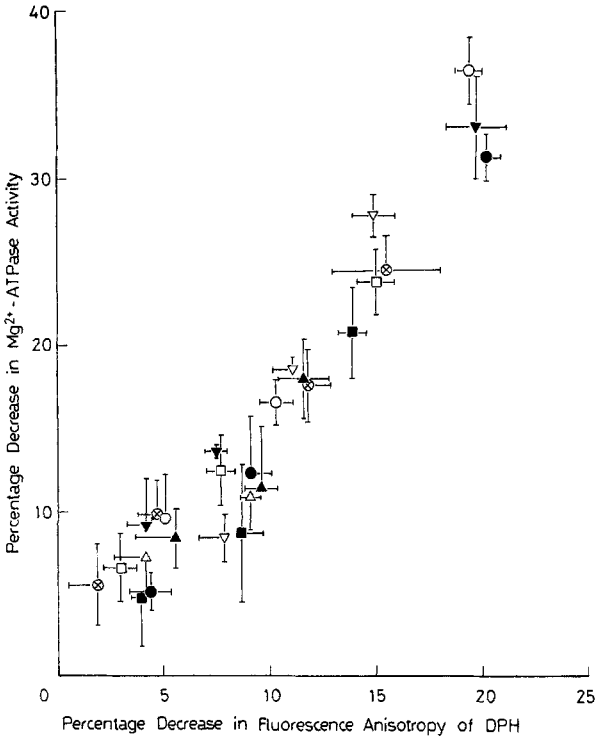

Fig. 4. Relationship between percentage decrease in fluorescence anisotropy of DPH in brush border membrane and percentage decrease in Mg^{2+}-ATPase activity induced by n-butyl (○), n-amyl (●), n-hexyl (⊗), n-heptyl (□), n-octyl (■), n-decyl (△), n-undecyl (▲), isoamyl (▽) and benzyl alcohol (▼). Values on fluorescence anisotropy, which are means ± SD for four experiments, were determined from the dose-dependent effects of the alcohols on the fluorescence anisotropy of DPH as shown in Fig. 2 for n-hexyl alcohol. Values on Mg^{2+}-ATPase activity were determined from the data in the Table.

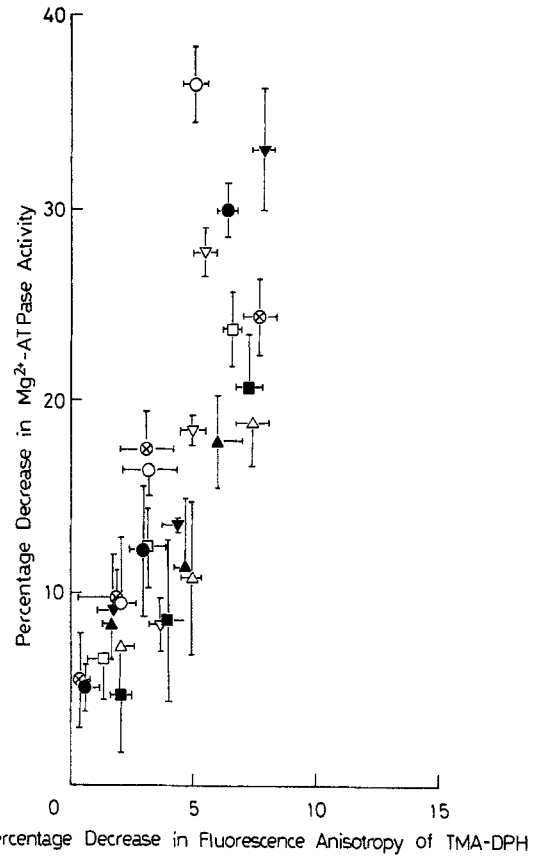

Fig. 5. Relationship between percentage decrease in fluorescence anisotropy of TMA-DPH in brush border membrane and percentage decrease in Mg^{2+}-ATPase activity induced by n-butyl (○), n-amyl (●), n-hexyl (⊗), n-heptyl (□), n-octyl (■), n-decyl (△), n-undecyl (▲), isoamyl (▽) and benzyl alcohol (▼). Values on fluorescence anisotropy, which are means ± SD for four experiments, were determined from the dose-dependent effects of the alcohols on the fluorescence anisotropy of TMA-DPH as shown in Fig. 2 for n-hexyl alcohol. Values of Mg^{2+}-ATPase activity were determined from the data in the Table.

ATPase activity by the alcohols was commonly correlated with the percentage decrease of fluorescence anisotropy of DPH. Furthermore, we examined the correlation with the effects on fluorescence anisotropy of ionic derivatives of DPH which are located in the different regions of the lipid bilayer. As shown in Fig. 5 for TMA-DPH, at the concentration range of the alcohols that decreased the fluorescence anisotropy by 5% or less, inhibition of the enzyme activity increased with the decrease in fluorescence anisotropy of TMA-DPH, although a correlation was not as clear as that observed for DPH. Beyond that range a correlation was not observed, especially for relatively hydrophilic alcohols. Because a good correlation was observed at all concentration ranges tested with the decrease in DPH, which is located in the central region of the lipid bilayer, it is possible that inhibition of Mg^{2+}-ATPase by the alcohols can simply be explained by a perturbation of that region.

Discussion

Alcohols have been found to affect the lipid phase transition (Jain et al., 1978), increase membrane fluidity (Kutchai, Cooper & Forster, 1980), provoke anesthesia (Pringle, Brown & Miller, 1981), modify various enzymatic activities (Mitjavila et al., 1976; Fernandez et al., 1984; Garda & Brenner, 1984; Kitagawa & Hirata, 1992), inhibit various transport processes (Devés & Krupka, 1990; Winpenny et al., 1992) and enhance flip-flop of phospholipids (Schwichtenhövel et al., 1992). Similar effects of n-alkyl alcohols with increasing alkyl chain length have been shown to occur at similar concentrations, and were also confirmed in this study. Inhibitory potencies of the alcohols on Mg^{2+}-ATPase activity and potencies of the same alcohols in decreasing the fluorescence anisotropies of DPH showed similar dependency on their partition coefficients, which suggests that partitioning amounts of the alcohols to the membrane also determine the inhibitory potencies on the ac-

tivity of the enzyme and the increases in membrane fluidity. The slopes in Eqs. (1) and (2), which represent the dependency on logarthmic values of their partition coefficients between n-octyl alcohol and water were similar for both effects, although the slope in Eq. (2) was only slightly larger than that in Eq. (1). The values of the slopes were also similar to those reported previously on hemolytic activities of the alcohols (C_3-C_9) in rabbit erythrocytes (Ponder & Hyman, 1939), inhibitory effects of the alcohols (C_3-C_6) on aggregation of bovine platelets (Kitagawa, Shinohara & Kametani, 1984) and abilities of the alcohols (C_3-C_8) to modify the order-disorder thermotropic transition in dipalmitoyl phosphatidylcholine, those to stimulate the hydrolysis of phosphatidylcholine in a bilayer by bee venom phospholipase A_2 and those to activate the galactoside transport system in *Escherichia coli* (Jain et al., 1978).

In this work, we clearly demonstrated for the alcohols, which differ in their alkyl chain lengths or chemical structures, that a common relationship was present, at a wide concentration range, between the inhibitory effects on Mg^{2+}-ATPase activities in rabbit small intestinal brush border membrane and the increases in membrane fluidity. To confirm this relationship, it would be necessary to observe other parameters on membrane fluidity such as order parameter of spin labels. However, the results obtained here suggest that inhibition of Mg^{2+}-ATPase by the alcohols might be explained by perturbation of the lipid bilayer of the brush border membrane, especially by perturbation of its central region. This possibility is consistent with the comparative effects of the alcohols on the various membrane functions mentioned above. Our speculation that alcohols inhibited Mg^{2+}-ATPase mainly by perturbation of the central region of the lipid bilayer also is in agreement with the previous findings on the effects of the alcohols in other membranes (Chin & Goldstein, 1985). However, other possibilities can not be excluded. For example, alcohols may affect the activity of Mg^{2+}-ATPase by direct perturbation of the membrane enzyme structure following binding to hydrophobic pockets in the enzyme, as suggested for inhibition of the activity of firefly luciferase (Dickinson, Franks & Lieb, 1993). Alcohols may affect the membrane enzyme by removal of lipids from the lipid-protein interface, as suggested for activation of Ca^{2+}-ATPase of sarcoplastic reticulum vesicles (Lopes & Louro, 1991) or by changing the dielectric constant (Orme et al., 1988).

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