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Inhibitory effects of long-chain alkyltrimethylammonium ions on aggregation of bovine platelets and the relation of their effects to Ca^{2+} mobilization

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The inhibitory effects of alkyltrimethylammonium ions on ADP- and thrombin-induced aggregation of bovine platelets were investigated. The ammonium cations inhibited the two aggregation reactions to similar extents. The relationship between their inhibitory effects on ADP-induced aggregation and their alkyl chain lengths from C_8 to C_{18} was investigated. Results showed that the inhibitory effects of ammonium cations increased with increase of their alkyl chain lengths up to C_{16}, and that the increase was linear with chain lengths of up to C_{14}. This linear relation and slope of the linear regression line suggested that the inhibitory effects of the ammonium cations depended on their partitioning into the membrane. However, unlike long-chain unsaturated fatty acids, they did not affect the membrane fluidity of the platelets. Fluorescence analysis of fura-2 loaded platelets revealed that, in the concentration range where the alkyltrimethylammonium ions inhibited aggregation, they inhibited agonist-induced increase in cytosolic Ca^{2+} both in the presence and absence of extracellular Ca^{2+}. These results suggest that inhibition of platelet aggregation by alkyltrimethylammonium ions is mainly due to their inhibition of increase in cytoplasmic Ca^{2+} by inhibition of both intracellular Ca^{2+} mobilization and Ca^{2+} uptake.

Introduction

Platelets are known to have essential roles in the manifestation of thrombosis and hemostasis through their abilities for adhesion, aggregation, and release of their granular contents [1,2]. Therefore, there have been many studies on the mechanisms of their reactions. Platelets have also frequently been used as models of biological cells in studies on the mechanisms of the effects of various physiological active agents, because they are easy to prepare, their clear physiological functions can readily be measured in vitro, and much is known about their membrane structures. A variety of reagents have been found to inhibit platelet functions in vitro [3–6], including drugs used clinically for prevention of thrombosis by inhibiting cyclooxygenase, such as aspirin [7] and activators of adenylate cyclase, such as prostaglandin I_2 [8]. Previously we reported that anionic and non-electrolytic amphiphilic fatty acids and alkyl alcohols also inhibit platelet functions [9–11]. Our results suggested that the inhibitory potencies are inhibitory mechanisms of these reagents depended on their physico-chemical characteristics, such as the presence of double bonds are electric charges.

In this work we examined the inhibitory effects of cationic amphiphilic alkyltrimethylammonium ions with various alkyl chain lengths and the relationship between their inhibitory effects and chain lengths. We also investigated their inhibi-

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tory mechanism in relation to change in membrane fluidity and Ca^{2+} mobilization. We focused on these problems because unsaturated fatty acids, which are also long-chain alkyl compounds, are suggested to modify platelet functions by causing membrane perturbation [11,12], whereas Ca^{2+} channel blockers such as verapamil and nifedipin, which are also ammonium ions, are suggested to inhibit platelet functions by inhibiting Ca^{2+} uptake [13,14].

Materials and Methods

Materials

ADP was purchased from the Oriental Yeast Co. (Tokyo, Japan). Bovine fibrinogen, bovine thrombin, diphenylhexatriene and oleic acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fura-2 acetoxy methyl ester was obtained from Dojindo Lab. (Kumamoto, Japan). Bromide salts of alkyltrimethylammonium ions were purchased from Tokyo Chemical Industries (Tokyo, Japan); these compounds were used as aqueous solutions in the experiments.

Measurement of aggregation

Platelet-rich plasma of bovine (Holstein) blood was obtained as described in a previous paper [28]. The platelet-rich plasma, which contained about 10% by volume of ACD anti-coagulant solution (74.8 mM sodium citrate, 38.1 mM citric acid and 122 mM dextrose), was centrifuged at $1000 \times g$ for 10 min and the platelets were suspended in a solution of 150 mM KCl/10 mM Tris-HCl adjusted to pH 7.4. Spontaneous platelet aggregation during preservation was prevented by adding 129 mM citrate (adjusted to pH 7.4) to this suspension at a volume ratio of 1 : 10. The platelet suspension was mixed with 9 vol. of KCl-Tris medium containing 1 mg/ml fibrinogen. The final platelet concentration was about $9 \cdot 10^4/\mu l$. For use in the assay of thrombin-induced aggregation, the platelets were suspended in the same medium but without fibrinogen. After preincubation with alkyltrimethylammonium ions for 2 min, 0.5 mM $CaCl_2$ and stimulants were added to the platelet suspension, and aggregation was measured at 37 °C in an RAM-11 aggregometer (Rikadenki Kogyo Co., Tokyo, Japan). The effects of alkyltrimethylammonium ions on aggregation were expressed as aggregation rates with the reagents relative to that without reagents as described previously [10,11].

Measurement of fluorescence polarization

Fluorescence polarization of diphenylhexatriene-labeled bovine platelets was measured as described previously [9–11]. Platelets at a concentration of about $9 \cdot 10^4/\mu l$ were incubated with 1.5 μM diphenylhexatriene for 40 min. Fluorescence in platelets was measured in a spectrofluorometer 650-40 (Hitachi Seisakusho Co., Tokyo, Japan) at 37 °C, with excitation and emission wavelengths of 362 and 428 nm, respectively. Fluorescence polarization was determined as described previously [10].

Measurement of cytoplasmic free calcium levels

The cytoplasmic concentration of free Ca^{2+} was measured as described by Grynkiewicz et al. [15] with the fluorescent dye fura-2. Platelets were loaded with fura-2 by incubating them in sodium/potassium-Tris medium (137 mM NaCl/5.4 mM KCl/11 mM dextrose/25 mM Tris-HCl adjusted to pH 7.4) containing 1 mM $MgCl_2$ for 30 min at 37 °C in the presence of 2 μM fura-2 acetoxy methyl ester. Then they were washed and suspended in KCl-Tris medium and the fluorescence change induced by ADP or thrombin either in the presence or absence of alkyltrimethylammonium ions was observed. Fura-2 fluorescence was measured at 37 °C in the spectrofluorometer described above with excitation and emission wavelengths of 339 and 500 nm, respectively. The intracellular Ca^{2+} concentration was calculated as described by Pollock et al. [16].

Results

Effects of alkyltrimethylammonium ions on platelet aggregation

First we examined the effects of tetradecyltrimethylammonium ion on bovine platelet aggregation induced by ADP and thrombin. As shown in Fig. 1, tetradecyltrimethylammonium ion inhibited the aggregations induced by these two compounds and the inhibitions were observed at similar concentration ranges of the ammonium ion

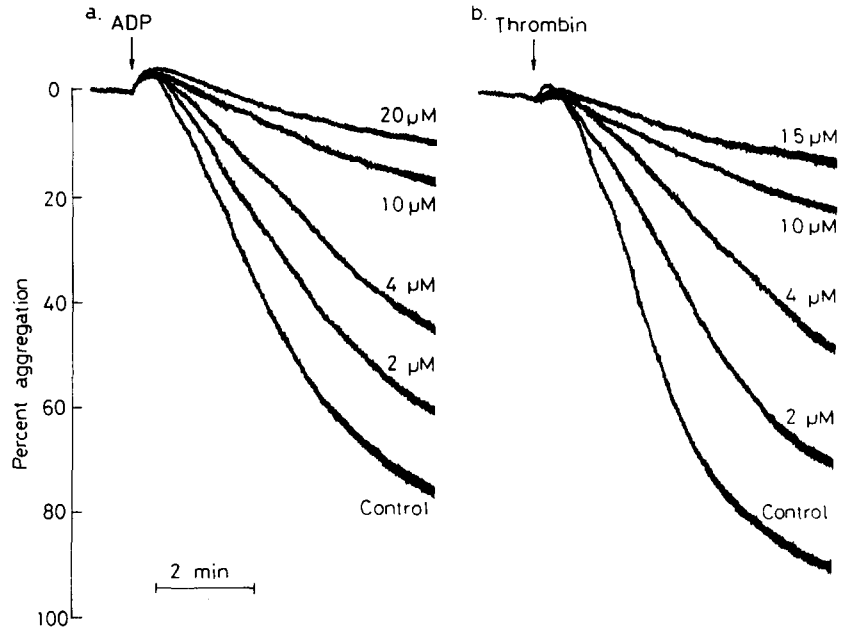

Fig. 1. Effects of tetradecyltrimethylammonium ion on platelet aggregation induced by 10 μM ADP (a) and 0.2 unit/ml thrombin (b). The concentrations of tetradecyltrimethylammonium ion added are shown in the figure. A platelet suspension obtained as described in the Materials and Methods ($9 \cdot 10^5$ platelets/μl) was mixed with 9 vol. of KCl-Tris medium either with (a) or without (b) 1 mg/ml fibrinogen. After addition of $CaCl_2$ at a final concentration of 0.5 mM, ADP or thrombin was added and aggregation was measured at 37° C. Aggregation was examined after a 2-min preincubation of the platelets with the ammonium ion.

under these conditions. Moreover, the aggregation rates and maximum aggregation in the two reactions were affected to similar extents. The inhibitions occurred at micromolar concentrations of the ammonium ion and, at a concentration of 4 μM, both aggregations were reduced by about half.

To determine the relation between the molecular features and inhibitory potencies of the ammonium ions, we then examined the inhibitory effects on ADP-induced aggregation of alkyltrimethylammonium ions of various alkyl chain lengths. The results are shown in Fig. 2, where inhibitory effects are expressed as ID_{50} values (i.e., concentrations inducing 50% inhibition of aggregation).

As shown in Fig. 2, the inhibitory effects of alkyltrimethylammonium cations increased with increase in their alkyl chain length up to C_{16}. The values for compounds with chain lengths of up to C_{14} fitted a straight line with a negative slope expressed by the following equation:

$$\log ID_{50}\ (\mu M) = -0.531\, n + 8.09 \quad (r = 1.000)$$

where n is the alkyl chain length of the ammonium ion and r is the correlation coefficient. The inhibitory effect of the compound C_{18} (stearyltrimethylammonium ion) was rather less than that of the C_{16} compound.

Effects of alkyltrimethylammonium ions on membrane fluidity

In an attempt to determine the mechanism of inhibition by alkylammonium ions, we next examined the effects of these compounds on membrane fluidity, because their analogs, such as alkyl alcohols and unsaturated fatty acids, seem to inhibit platelet aggregation by causing membrane perturbation [9,11]. We examined effects on fluidity as effects on fluorescence polarization of diphenylhexatriene-labeled platelets. As shown in Table I, addition of decyltrimethylammonium ion (C_{10}) or tetradecyltrimethylammonium ion (C_{14}) did not affect fluorescence polarization, indicating that these compounds did not influence membrane fluidity, even at concentrations that inhibited aggregation almost completely. Similar results were obtained with other alkyltrimethylam-

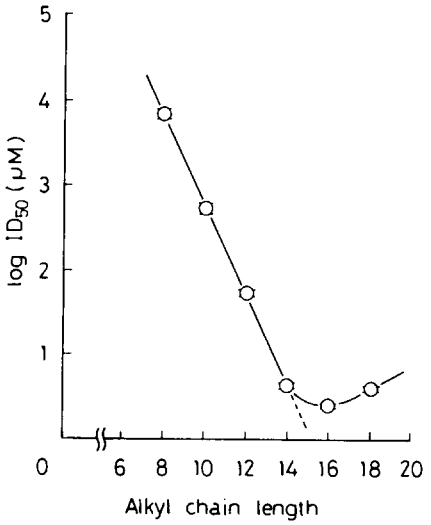

Fig. 2. Relation between the logarithms of the 50% inhibitory concentrations ID_{50} values, of alkyltrimethylammonium ions, on 10 μM ADP-induced aggregation and their alkyl chain lengths. Values are means ± S.D. for three experiments.

monium ions. This result is in contrast to results with long-chain unsaturated fatty acids such as oleic acid, the effect of which is also shown in Table I, which did influence membrane fluidity.

Effects of alkyltrimethylammonium ions on the increase in the cytoplasmic Ca^{2+} concentration

We then examined the effects of alkyltrimethylammonium ions on the increase in the cytoplasmic Ca^{2+} concentration induced by thrombin and ADP, because this increase in cytoplasmic Ca^{2+} concentration is essential for platelet activation [17,18], and because several reagents such as prostacyclin are reported to inhibit platelet functions by inhibiting this increase [19–21]. As shown in Fig. 3, thrombin induced the increase in the cytoplasmic Ca^{2+} concentration either in the presence (Fig. 3a) or absence (Fig. 3b) of extracellular Ca^{2+}, but the increase was more rapid and greater in the presence of extracellular Ca^{2+} than in its absence. This result is consistent with that reported in human platelets [27]. Alkyltrimethylammonium ions inhibited this increase in cytosolic Ca^{2+} dose dependently both in the presence and absence of extracellular Ca^{2+}, as shown in Fig. 3 for tetradecyltrimethylammonium ion. The concentration ranges of ammonium ions for this inhibition in the presence of extracellular Ca^{2+} were similar to those for their inhibition of aggregation shown in Fig. 1. Similar results were obtained with the effects of alkyltrimethylammonium ions on the ADP-induced increase in the cytoplasmic Ca^{2+}

TABLE I

EFFECTS OF ALKYLTRIMETHYLAMMONIUM IONS ON FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE-LABELED PLATELETS

Fluorescence polarization of diphenylhexatriene-labeled platelets was measured as described previously [9–11] after a 2-min preincubation of platelets with the ammonium ions. Data are means ± S.D. for five to eight experiments. Data on oleic acid are also listed.

Reagent	Concentration	Fluorescence polarization
Control		0.230 ± 0.005
Decyltrimethylammonium ion (mM)	1	0.229 ± 0.006
	2	0.232 ± 0.004
Tetradecyltrimethylammonium ion (μM)	10	0.232 ± 0.005
	20	0.235 ± 0.004
Oleic acid (μM)	10	0.212 ± 0.010

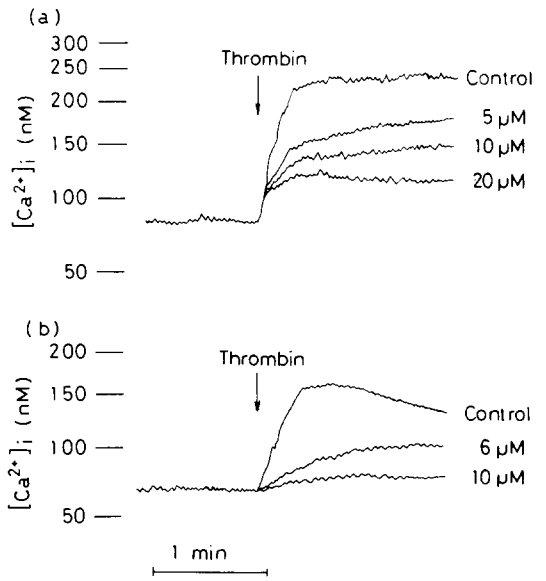

Fig. 3. Effects of tetradecyltrimethylammonium ion on the thrombin-induced rise in cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$ in the presence of 0.5 mM external $CaCl_2$ (a) and in the presence of 1 mM EDTA with no added $CaCl_2$ (b). The concentrations the ammonium ion used are shown in the figure. After a 2-min preincubation of the platelets with the ammonium ion at 37 °C, thrombin was added at 0.2 unit/ml and fluorescence changes were monitored.

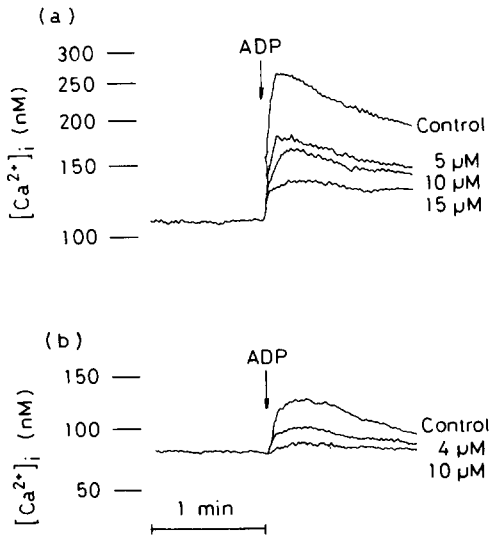

Fig. 4. Effects of tetradecyltrimethylammonium ion on the 10 μM ADP-induced rise in cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$ in the presence of 0.5 mM external $CaCl_2$ (a) and in the presence of 1 mM EDTA with no added $CaCl_2$ (b). The concentrations the ammonium ion used are shown in the figure. The experimental procedure was as for Fig. 3.

concentration, as shown in Fig. 4 for tetradecyltrimethylammonium ion.

Discussion

Various amphiphilic compounds have been reported to inhibit platelet aggregation. We have reported that, of the long-chain alkyl compounds tested, unsaturated fatty acids such as linoleic acid inhibit both ADP- and thrombin-induced platelet aggregation appreciably, whereas saturated fatty acids with chain lengths of up to C_{16} inhibit ADP-induced aggregation [9] but cause much less inhibition of thrombin-induced aggregation. Here we report that cationic long-chain alkyltrimethylammonium ions inhibit the aggregations induced by ADP and thrombin to similar extents. Their inhibitory concentrations were below their critical micelle concentrations and below their concentrations causing lysis of platelets, just like those of unsaturated fatty acids. Moreover, we observed a linear relationship between the inhibitory effects of alkyltrimethylammonium ions on platelet aggregation and their alkyl chain lengths with chain lengths of up to C_{14}. Previously we found a similar linear relationship between the inhibitory effects on ADP-induced aggregation of long-chain saturated fatty acids and their alkyl chain lengths with chain lengths of up to C_{12} [9]. As with saturated fatty acids, this linear relation for alkyltrimethylammonium ions and its slope suggest that the inhibitory effects depend on their partition into the membrane. Therefore, we suspect that ammonium cations exert their effects by partitioning into the membrane layer, as in their inhibition of the sodium pump of red cells, for which a similar linear relationship has been observed [22] and in their antihemolytic action [23] and, at higher concentrations, their hemolytic action [22].

The inhibitory effects of the alkylammonium ions with chain lengths of more than C_{14} deviated from this linear regression, and the effect of the C_{18} compound tended to be less than that of the C_{16} compound. Similar tendencies were also found for sodium pump inhibition by the same compounds [22] and for aggregation inhibition by saturated fatty acids [9]. This relationship was probably due to a difference in the extents of partitioning of these compounds into the membrane, as demonstrated for saturated fatty acids in the erythrocyte membrane [24]. As suggested by Klein and Ellory [22], there may be a physical effect of the length of the alkyl chain, and kinking of a very long alkyl chain may be necessary for its entry into the ordered acyl chains of membrane phospholipids.

Although the inhibitory actions of the alkylammonium ions seem to be exerted through interaction with the platelet membrane, the mechanism of interaction may be different from that of long-chain unsaturated fatty acids, because inhibition of platelet aggregation by unsaturated fatty acids is associated with increase in membrane fluidity, whereas that by alkyltrimethylammonium ions is not. Saturated fatty acids which are like alkyltrimethylammonium ions were also found to have little influence on the membrane fluidity of the platelets [9,11]. Therefore, *cis*-unsaturated bonds in long-chain alkyl compounds may be essential for effects on membrane fluidity.

In this work we found that, in the concentration ranges for their inhibition of aggregation, alkylammonium ions inhibited increase in cytosolic Ca^{2+} induced by either thrombin or ADP.

Thus inhibition of aggregation by alkyltrimethylammonium ions seems to be mainly due to inhibition of this increase in cytosolic Ca^{2+}. This inhibition of increase on Ca^{2+} occurred in both the presence and absence of extracellular Ca^{2+}, so these compounds probably inhibit both intracellular Ca^{2+} mobilization and Ca^{2+} uptake. It is unknown whether these ammonium cations inhibit increase in cytosolic Ca^{2+} by inhibiting the binding of agonists or inhibiting some process that triggers intracellular Ca^{2+} release or Ca^{2+} uptake by modifying some enzymatic process. One possible candidate as an enzymatic process is the inositol phospholipid metabolism, since a metabolite formed in this process, inositol-1,4,5-triphosphate (IP_3), is suggested to trigger Ca^{2+} release from intracellular pools [25], and an alkyl polyamine, spermine, was reported to inhibit thrombin-induced phosphatidylinositol metabolism [26]. Further studies on the mechanism of action of these ammonium cations are in progress.

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