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Effects of long-chain *cis*-unsaturated fatty acids and their alcohol analogs on aggregation of bovine platelets and their relation with membrane fluidity change

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The effects of long-chain *cis*-unsaturated fatty acids with different alkyl chain lengths and different numbers of double bonds on aggregation of bovine platelets and membrane fluidity were investigated. All the *cis*-unsaturated fatty acids tested inhibited aggregation and at the same time increased membrane fluidity in accordance with their inhibitory effects. The saturated fatty acids and *trans*-unsaturated fatty acid tested for comparison had much lower or no effects on aggregation and membrane fluidity. The inhibitory effects of mono *cis*-unsaturated fatty acids increased with increase of their alkyl chain length. *cis*-Unsaturated fatty acids with two or more double bonds had more inhibitory effects than mono-unsaturated fatty acids. The position of the double bonds had less influence than the number of double bonds. We also examined the effects of *cis*-unsaturated fatty acids on membrane fluidity with diphenylhexatriene and anthroyloxy derivatives of fatty acids as probes and observed increased fluidity to be considerable in the membrane. The alcohol analogs of *cis*-unsaturated fatty acids also inhibited aggregation and increased membrane perturbation. These results suggest that the inhibition of platelet aggregation by *cis*-unsaturated compounds is due to perturbation of the lipid layer.

Introduction

Long-chain fatty acids, which are components of phospholipid acyl chains, are known to regulate membrane viscosity as cholesterol does [1,2]. Recently the fluidity of the plasma membrane was suggested to be important in platelet functions. Shattil et al. [3,4] observed increased sensitivity of cholesterol-rich platelets to aggregating agents and its correlation with reduction of membrane fluidity. There are also many reports that long-chain *cis*-unsaturated fatty acids, such as linoleic acid, at concentrations well below their critical micelle concentrations inhibit platelet aggregation induced by ADP or thrombin [5–7]. Their inhibitory effects of these fatty acids also seem to be at least partly related with their effects in causing membrane perturbation [6,7].

In this work we examined the inhibitory effects of various long-chain *cis*-unsaturated fatty acids, and particularly the relations between the inhibitory effects of the fatty acids and their alkyl chain lengths and numbers of double bonds. We also examined the effects of some of their alcohol analogs, whose effects on platelet aggregation are unknown. Moreover, we investigated the effects of these compounds on fluidity of platelet plasma membranes with diphenylhexatriene and anthroyloxy derivatives of long-chain fatty acids as fluorescent probes to determine the fluidity changes in various domains of the membranes [8,9]. From the results, we discuss the relation between membrane

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fluidity change and inhibition of aggregation by these compounds.

Materials and Methods

Materials. Fatty acids, alkyl alcohols, bovine fibrinogen and diphenylhexatriene were purchased from Sigma Chemical Co. (St. Louis, MO). 2-(9-Anthroyloxypalmitate) and 12-(9-anthroyloxystearate) were purchased from Molecular Probes, Inc. (Junction City, OR). ADP was from Oriental Yeast Co. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fatty acids and alkyl alcohols were prepared as 100 mM solutions in methanol just before use, because methanol itself had no effect on both aggregation and fluorescence polarization at concentrations under 15 mM used here.

Measurement of aggregation. Aggregation of bovine (Holstein) blood platelets was measured as described previously [6,10]. That is, a suspension of platelets separated from plasma protein by centrifugation was mixed with 9 vol. of Na,K-Tris medium (137 mM NaCl, 5.4 mM KCl, 11 mM dextrose, 25 mM Tris-HCl adjusted to pH 7.4) containing 1 mg/ml fibrinogen and fatty acids or alkyl alcohols; the final platelet concentration was about $9 \cdot 10^4/\mu l$. After addition of $CaCl_2$ at a final concentration of 0.5 mM, the platelet suspension was preincubated with fatty acids or alkyl alcohols for 2 min. Then ADP was added and aggregation was measured at 25°C unless otherwise mentioned by recording the absorbance at 600 nm in a spectrophotometer UV-180 (Shimadzu 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 reagents on aggregation were expressed as aggregation rates with the reagents relative to that without reagents as described previously [6,11].

Measurement of fluorescence polarization. Fluorescence polarization of diphenylhexatriene- or 9-anthroyloxy derivatives of fatty acid-labeled bovine platelets was measured as described previously [6,10,11]. Platelets at a concentration of about $9 \cdot 10^4/\mu l$ were incubated with 1 μM diphenylhexatriene, or 1.5 μM 2-(9-anthroyloxypalmitate) and 12-(9-anthroyloxystearate) for 40 min. These cocentrations of probes had no effects on platelet aggregation. Fluorescence in platelets was measured in a spectrofluorometer 650-40 (Hitachi Seisakusho Co., Tokyo, Japan) at 25°C. The excitation and emission wavelengths used with diphenylhexatriene, 2-(9-anthroyloxypalmitate) and 12-(9-anthroyloxystearate) were 360 and 428 nm, 370 and 438 nm, and 368 and 443 nm, respectively. Fluorescence polarizaation was determined as described previously [6,11].

Results

Effects of long-chain cis-unsaturated fatty acids on platelet aggregation

First we examined the effects of nine long-chain unsaturated fatty acids with 1 to 5 double bonds on ADP-induced aggregation at 25°C. For comparison, we also investigated the effects of three long-chain saturated fatty acids. The aggregation was not inhibited by the three saturated fatty acids or the *trans*-unsaturated fatty acid elaidic acid at concentrations of less than 30 μM, but was inhibited by all the *cis*-unsaturated fatty acids tested. As shown in Fig. 1, oleic, linoleic and linolenic acid affected the aggregation rate and the maximum aggregation to similar degrees. The results listed in Table I and Fig. 1 show that unsaturated fatty acids with two or more double bonds were much more inhibitory than mono-unsaturated fatty acids with the same chain length. There was little difference in the inhibitory effects of di- and tri-unsaturated fatty acids. The order of the inhibitory effects of *cis*-mono-unsaturated fatty acids with different chain lengths was myristoleic < palmitoleic < oleic, indicating increase of inhibition with increase in alkyl chain length. Results with $C_{18:1}$ mono-unsaturated fatty acids indicated that the effect of the position of double bonds was less than that of numbers of double bonds.

We also investigated the effects of these fatty acids on platelet aggregation induced by other compounds and obtained similar results. As shown in Fig. 2 for the effects of linoleic acid, *cis*-unsaturated fatty acids inhibited thrombin-induced aggregation of bovine platelets. They also inhibited collagen-induced aggregation similarly to ADP-induced one (data not shown).

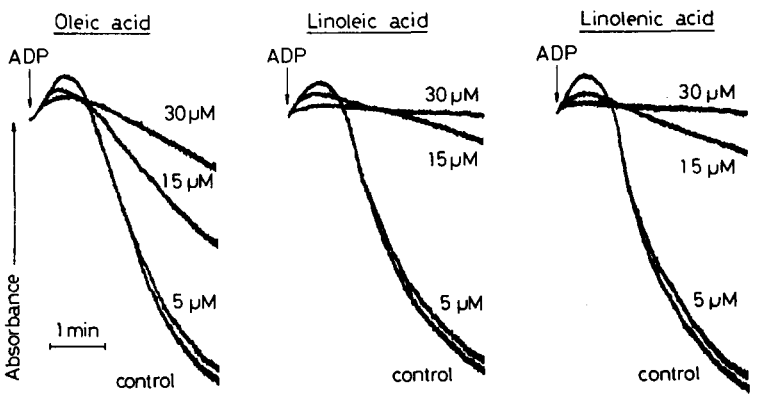

Fig. 1. Effects of oleic, linoleic and linolenic acid on 5 μM ADP-induced aggregation. The concentrations of the acids are shown in the figure. A platelet suspension obtained as described in Materials and Methods (concn. $9 \cdot 10^5/\mu l$) was mixed with 9 vol. of Na,K-Tris medium containing 1 mg/ml fibrinogen. After addition of $CaCl_2$ at a final concentration of 0.5 mM, ADP was added and aggregation was measured at 25°C. Aggregation was examined after 2-min preincubation of the platelets with the acids.

At higher temperature, the saturated fatty acid myristic acid also inhibited aggregation. As reported previously, all saturated fatty acids except stearic acid inhibited aggregation [12]. Saturated fatty acids mainly affected maximum aggregation, as shown in Fig. 3 for myristic acid. However, their inhibitory effects were much less than those of their unsaturated analogs at the same temperature.

Effects of long-chain cis-unsaturated fatty acids on fluorescence polarization of diphenylhexatriene- and anthroyloxy fatty acid-labeled platelets

The long-chain unsaturated fatty acids oleic and linoleic acid have been suggested to inhibit platelet aggregation in vitro by causing membrane perturbation [6,7]. We examined the effects of unsaturated fatty acids on the fluidity of platelet plasma membranes with diphenylhexatriene as

TABLE I

EFFECTS OF 30 μM LONG-CHAIN FATTY ACIDS ON 5 μM ADP-INDUCED AGGREGATION AND FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE-LABELED PLATELETS AT 25°C

Aggregation was examined after 2-min preincubation of platelets with the acids. Aggregation rate in the absence of the acids was defined as 1.00. Fluorescence polarization was measured as described previously [6,11]. Data on aggregation are means ± S.D. for three experiments and data on fluorescence polarization are those for five to eight experiments.

Acid	Structure	Relative aggregation rate	Fluorescence polarization
Control	–	1.00	0.238 ± 0.002
Myristic	$C_{14:0}$	1.10 ± 0.10	0.231 ± 0.011
Myristoleic	$C_{14:1}$(*cis*-9)	0.78 ± 0.07	0.224 ± 0.006
Palmitic	$C_{16:0}$	0.98 ± 0.06	0.234 ± 0.016
Palmitoleic	$C_{16:1}$(*cis*-9)	0.45 ± 0.01	0.225 ± 0.006
Stearic	$C_{18:0}$	0.92 ± 0.10	0.233 ± 0.005
Elaidic	$C_{18:1}$(*trans*-9)	1.12 ± 0.02	0.234 ± 0.004
Petroselinic	$C_{18:1}$(*cis*-6)	0.40 ± 0.03	0.213 ± 0.004
Oleic	$C_{18:1}$(*cis*-9)	0.23 ± 0.01	0.201 ± 0.008
Vaccenic	$C_{18:1}$(*cis*-11)	0.29 ± 0.04	0.216 ± 0.002
Linoleic	$C_{18:2}$(*cis*-9,12)	0.03 ± 0.01	0.207 ± 0.007
Linolenic	$C_{18:3}$(*cis*-9,12,15)	0.05 ± 0.01	0.200 ± 0.016
Eicosapentaenoic	$C_{20:5}$(*cis*-5,8,11,14,17)	0.07 ± 0.03	0.206 ± 0.013

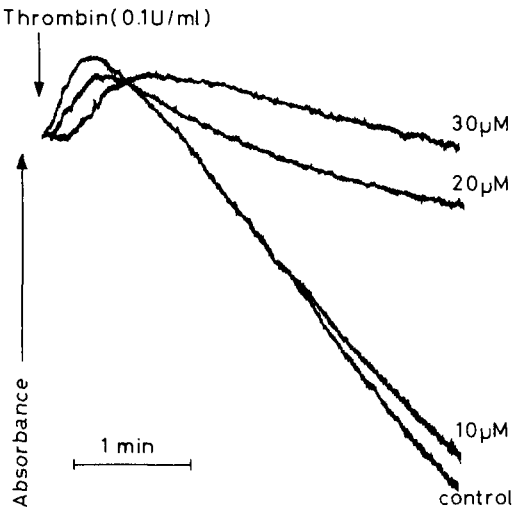

Fig. 2. Effect of linoleic acid on 0.1 U/ml thrombin-induced aggregation. The concentrations of the acid are shown in the figure. Aggregation was examined at 37°C in the absence of fibrinogen. Experimental procedure was similar to that described in Fig. 1.

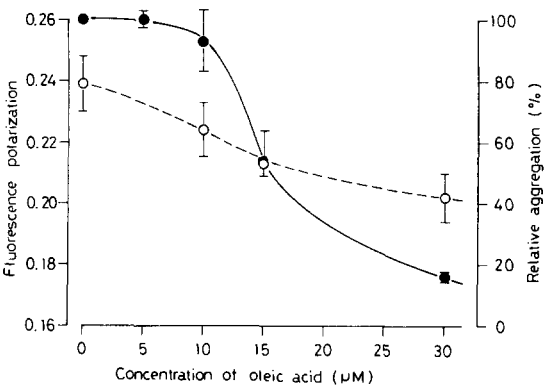

Fig. 4. Effects of oleic acid on fluorescence polarization of diphenylhexatriene-labeled platelets (○------○) and 5 μM ADP-induced aggregation (●———●). Fluorescence intensities of 1 μM diphenylhexatriene-labeled platelets at 25°C were detected through a polarizer oriented parallel and perpendicular to the direction of the polarized excitation beam in the presence or absence of the fatty acid. Fluorescence polarization was calculated as described previously [6,11]. The effect of oleic acid on aggregation was examined at 25°C after 2-min preincubation of platelets with the acid using same preparations of platelets. Data on fluorescence polarization are means ± S.D. for five experiments and data on aggregation are those for three experiments.

probe. As shown in Table I, all the *cis*-unsaturated fatty acids decreased the fluorescence polarization significantly, indicating increase in membrane fluidity. In contrast, saturated fatty acids and the *trans*-unsaturated fatty acid elaidic acid, which did not inhibit the aggregation, had no effect on fluorescence polarization. Moreover, as shown in Fig. 4 for oleic acid and reported previously for linoleic acid [5], the decrease in fluorescence polarization by *cis*-unsaturated fatty acids varied with their inhibitory effects in the concentration range that inhibited aggregation.

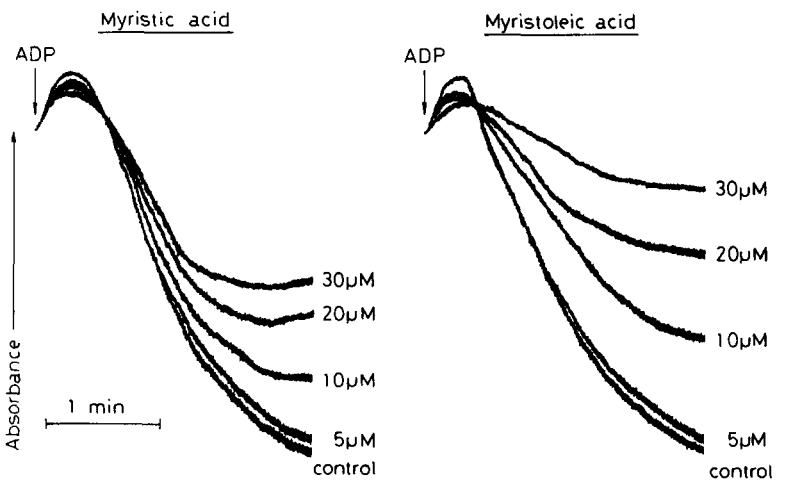

Fig. 3. Effects of myristic and myristoleic acid on 5 μM ADP-induced aggregation at 37°C. Experimental procedures were as for Fig. 1 except for the temperature.

TABLE II

EFFECTS OF 30 μM LONG-CHAIN FATTY ACIDS ON FLUORESCENCE POLARIZATION OF 2-(9-ANTHROYLOXYPALMITATE)- OR 12-(9-ANTHROYLOXYSTEARATE)-LABELED PLATELETS AT 25°C

Fluorescence intensities of 2-(9-anthroyloxypalmitate)-, 2-AP-, or 12-(9-anthroyloxystearate)-, 12-AS-, labeled platelets were measured by the same method as those of diphenylhexatriene-labeled platelets. Data are means ± S.D. for three to five experiments.

Probe	Acid	Fluorescence polarization
2-AP	Control	0.168 ± 0.005
	Palmitic	0.163 ± 0.004
	Elaidic	0.166 ± 0.009
	Linoleic	0.135 ± 0.003
	Linolenic	0.138 ± 0.006
12-AS	Control	0.090 ± 0.004
	Elaidic	0.091 ± 0.005
	Oleic	0.073 ± 0.001
	Linoleic	0.077 ± 0.002

In further studies on the effects of *cis*-unsaturated fatty acids on membrane fluidity, we examined the effects of these acids on 2-(9-anthroyloxypalmitate)- or 12-(9-anthroyloxystearate)-labeled platelets. These anthroyloxy fatty acids have been used in studies on the effects of various reagents on different levels in the lipid bilayer [9] and on the fluidity and polarity gradients in the lipid bilayers [8,9,13]. 2-(9-Anthroyloxypalmitate) is a probe of the fluidity relatively near the surface of the membrane lipid bilayer, and 12-(9-anthroyloxystearate) a probe of fluidity near the center of the lipid bilayer.

As shown in Table II, the fluorescence polarization of 2-(9-anthroyloxypalmitate)-labeled platelets was much higher than that of 12-(9-anthroyloxystearate)-labeled platelets. These results are consistent with those on phospholipid liposomes [9]. *cis*-Unsaturated fatty acids such as linoleic acid that inhibited aggregation decreased fluorescence polarization of 2-(9-anthroyloxypalmitate) and 12-(9-anthroyloxystearate), whereas saturated fatty acids and a *trans*-unsaturated fatty acid had no effects. These results suggest that *cis*-unsaturated fatty acids increased membrane fluidity to a considerable depth in the membrane lipid layer.

Effects of long-chain unsaturated alkyl alcohols on ADP-induced aggregation and membrane fluidity

The above results suggested a relation between inhibition of aggregation and change in membrane fluidity by unsaturated fatty acids. To determine whether this was also true with other unsaturated compounds, we next examined the effects of long-chain unsaturated alkyl alcohols on platelet aggregation and membrane fluidity. We examined the effects of the three alcohols listed in Table III, because the solubilities of these alcohols are relatively high. When tested at 25°C, of these three alcohols, only arachidonyl alcohol at concentrations of less than 30 μM inhibited aggregation and also markedly decreased the fluorescence polarization of diphenylhexatriene-labeled platelets (Table III). At higher temperature, linolenyl alcohol at higher concentrations also inhibited aggregation and decreased fluorescence polarization significantly (Table IV).

TABLE III

EFFECTS OF 30 μM LONG-CHAIN ALKYL ALCOHOLS ON 5 μM ADP-INDUCED AGGREGATION AND FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE-LABELED PLATELETS AT 25°C

Relative aggregation rate and fluorescence polarization were determined as described for Table I. Data on aggregation are means ± S.D. for three experiments and data on fluorescence polarization are those for five to eight experiments.

Alcohol	Structure	Relative aggregation rate	Fluorescence polarization
Control	–	1.00	0.238 ± 0.002
Linoleyl	$C_{18:2}$(*cis*-9,12)	1.13 ± 0.02	0.234 ± 0.005
Linolenyl	$C_{18:3}$(*cis*-9,12,15)	0.94 ± 0.02	0.233 ± 0.006
Arachidonyl	$C_{20:4}$(*cis*-5,8,11,14)	0.62 ± 0.05	0.197 ± 0.005

TABLE IV

EFFECTS OF LINOLENYL ALCOHOL ON AGGREGATION AND FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE-LABELED PLATELETS AT 25°C AND 37°C

Relative aggregation rate and fluorescence polarization were determined as described for Table I. Data on aggregation are means ± S.D. for three experiments and data on fluorescence polarization are those for five to eight experiments. The aggregation rate in the absence of linolenyl alcohol at each temperature was defined as 1.00.

Temp. (°C)	Concn. (μM)	Decrease of fluorescence polarization	Relative aggregation rate
25	30	0.005 ± 0.007	0.94 ± 0.02
25	50	0.006 ± 0.010	0.91 ± 0.02
37	30	0.009 ± 0.003	0.85 ± 0.09
37	50	0.029 ± 0.005	0.64 ± 0.03

Discussion

In this work we found that all the *cis*-unsaturated fatty acids tested inhibited platelet aggregation and also increased membrane fluidity in accordance with their inhibitory effects on aggregation, as shown previously for oleic and linoleic acid [6,7]. On the other hand, saturated fatty acids and *trans*-unsaturated fatty acid had little or no effects on membrane fluidity or aggregation. The results on membrane fluidity are consistent with the findings that *cis*-unsaturated fatty acids increase membrane fluidity of a phospholipid membrane and other biological membranes, whereas saturated fatty acids and *trans*-unsaturated fatty acids do not [13]. These results suggest that, unlike saturated fatty acids, which seem to inhibit aggregation at least partly by changing the membrane surface charge [12], *cis*-unsaturated fatty acids in general inhibit platelet aggregation because their *cis*-unsaturated bonds cause membrane perturbation. This conclusion is supported by the finding that *cis*-poly-unsaturated fatty acids had more effect than *cis*-mono-unsaturated fatty acids. The membrane partitions of acids with two or more double bonds seem to be less than those of mono-unsaturated fatty acids, because these acids are relatively more hydrophilic. However, since they cause much more perturbation than the same amount of mono-unsaturated fatty acids, they seem to cause a larger increase in the membrane fluidity. The fact that there is little difference in the effects of unsaturated fatty acids with two or more double bonds may be due to the balance of their membrane partition and membrane perturbing effects.

We also observed an increase in the inhibitory effects of *cis*-9 mono-unsaturated fatty acids with increase in their alkyl chain lengths. This was probably due to the difference in membrane partitions of these acids [14]. In fact the decreases of fluorescence polarization caused by myristoleic acid and palmitoleic acid were slightly less than that caused by oleic acid. Our results also showed that the position of the double bond in mono-unsaturated fatty acids had less influence on the effects of these compounds on membrane fluidity and platelet functions.

Inhibition of phospholipase A_2 [15] or cyclooxygenase [16] has been postulated as a mechanism of inhibition of platelet functions by long-chain *cis*-unsaturated fatty acids such as linoleic acid. However, these possibilities seem improbable because the unsaturated fatty acids caused similar inhibitions of the aggregation induced by various kinds of stimulants, irrespective of whether the stimulant activities were dependent on arachidonate metabolism, and because the acids also inhibit aggregation of platelets in which cyclooxygenase activity had been inhibited by indomethacin [15,17]. Since the effects of *cis*-unsaturated fatty acids in general on platelet aggregation and membrane fluidity were parallel, as revealed in this work, it is more probable that these acids inhibit aggregation by causing membrane perturbation. This hypothesis is supported by recent studies on the effects of cholesterol and cations suggesting that a relatively rigid membrane favors platelet aggregation [5,18]. Our results with anthroyloxy derivatives of long-chain fatty acids indicated that the acids caused perturbation to a considerable depth in the membrane, although because the anthracene rings of the probes were large, this technique provides only average values for some depths [9].

The *cis*-unsaturated fatty acid eicosapentaenoic acid has been reported to modify platelet functions by competing with arachidonic acid as a

substrate of cyclooxygenase [19,20]. However, it also seems to affect platelet functions, at least partly, by causing membrane perturbation, especially in bovine platelets in which arachidonate metabolism is low [21].

The alcohol analogs of *cis*-unsaturated fatty acids also seemed to modify platelet functions by a similar mechanism, because they caused both aggregation inhibition and increase in membrane fluidity. However, relative to the acids, they had less effect on aggregation than on membrane fluidity. Therefore, the interaction of the unsaturated compounds with the boundary lipid layer, where unsaturated fatty acids probably interact more than their alcohol analogs with phospholipids, may be especially important for aggregation inhibition. The inhibitory effects of the alcohols were much less than those of the acids, but this is not peculiar to platelet aggregation, since it is also the case in their effects on osmotic fragility of erythrocytes [22]. This difference in the inhibitory effects of the acids and alcohols may reflect a difference in their binding to the membrane.

It is still unknown why membrane perturbaiton should affect platelet aggregation. Membrane fluidity is suggested to modify various membrane associated functions such as enzymatic activities [23] and ionic transport [24]. Moreover, a change in membrane fluidity is suggested to affect the cytoskeletal network because cytoskeleton interacts with the lipid layer [25]. Since reorganization of cytoplasmic contractile and structure proteins is essential for platelet functions [26,27], perturbation of the lipid layer by *cis*-unsaturated compounds may inhibit platelet aggregation by modifying this reorganization.

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References

1 De Gier, J., Mandersloot, J.G. and Van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 150, 666–675
2 Stubbs, C.D. and Smith, A.D. (1984) Biochim. Biophys. Acta 779, 89–137
3 Shattil, S.J., Anaya-Galindo, R., Bennett, J., Colman, R.W. and Cooper, R.A. (1975) J. Clin. Invest. 55, 636–643.
4 Shattil, S.J. and Cooper, R.A. (1976) Biochemistry 15, 4832–4837
5 Hoak, J.C., Spector, A.A., Fry, G.L. and Warner, E.D. (1970) Nature 228, 1330–1332
6 Kitagawa, S., Endo, J. and Kametani, F. (1984) Biochim. Biophys. Acta 798, 210–215
7 MacIntyre, D.E., Hoover, R.L., Smith, M., Steer, M., Lynch, C., Karnovsky, M.J. and Salzman, E.W. (1984) Blood 63, 848–857
8 Tilley, L., Thulborn, K.R. and Sawyer, W.H. (1979) J. Biol. Chem. 254, 2592–2594
9 Kutchai, H., Chandler, L.H. and Zavoico, G.B. (1983) Biochim. Biophys. Acta 736–149
10 Kitagawa, S., Endo, J. and Kametani, F. (1984) Biochim. Biophys. Acta 802, 17–23
11 Kitagawa, S., Shinohara, T. and Kametani, F. (1984) J. Membrane Biol. 79, 97–102
12 Kitagawa, S., Nishitama, H. and Kametani, F. (1984) Biochim. Biophys. Acta 775, 197–202
13 Klausner, R.D., Kleinfeld, A.M., Hoover, R.L. and Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286–1295
14 Sallee, V.L. (1978) J. Membrane Biol. 43, 187–201
15 Vallee, E., Gougat, J. and Ageron, M. (1980) Agents Actions 10, 57–62
16 Needleman, S.W., Spector, A.A. and Hoak, J.C. (1982) Prostaglandins 24, 607–621
17 MacIntyre, D.E., Hoover, R.L., Karnovsky, M.J. and Salzman, E.W. (1981) Thromb. Haemostas. 46, 277 (Abstr.)
18 Sauerheber, R.D., Zimmerman, T.S., Esgate, J.A., VanderLaan, W.P. and Gordon, L.M. (1980) J. Membrane Biol. 52, 201–218
19 Dyerberg, J. and Bang, H.O. (1978) Lancet i, 152
20 Gryglewski, R.J., Salmon, J.A. Ubatuba, F.B., Weatherly, B.C., Moncada, S. and Vane, J.R. (1979) Prostaglandins 18, 453–478
21 Meyers, K.M., Katz, J.B., Clemmons, R.M., Smith, J.B. and Holmsen, H. (1980) Thromb. Res. 20, 13–24
22 Raz A. and Livne, A. (1973) Biochim. Biophys. Acta 311, 222–229
23 Hirata, F. and Axelrod, J. (1980) Science 209, 1082–1090
24 Deuticke, B. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 1–97
25 Noji, S., Takahashi, T. and Kon, H. (1982) Biochem. Pharmacol. 31, 3178–3180
26 Jennings, L.K., Fox, J.E.B., Edwards, H.H. and Phillips, D.R. (1981) J. Biol. Chem. 256, 6927–6932
27 Pribluda, V. and Rotman, A. (1982) Biochemistry 21, 2825–2832