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AJOENE, THE ANTIPLATELET COMPOUND DERIVED FROM GARLIC, SPECIFICALLY INHIBITS PLATELET RELEASE REACTION BY AFFECTING THE PLASMA MEMBRANE INTERNAL MICROVISCOSITY

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Abstract—Ajoene (E,Z-4,5,9-trithiadodeca-1,6,11-triene 9-oxide), a product of the rearrangement of allicin (a major component of raw garlic), has been shown to be a potent inhibitor of platelet aggregation *in vitro* through inhibition of granule release and fibrinogen binding. Our present study further elaborates on this inhibitory action, through studies of the effect of ajoene on the earliest steps of platelet activation. The transducing mechanism involved in thrombin-induced platelet activation was not modified by the drug as indicated by a normal breakdown of phosphatidylinositol 4,5,bisphosphate and normal production of phosphatidic acid. Likewise, the agonist-induced phosphorylation of myosin light chain (P20) and of the 43 kD protein (P43) were not impaired by ajoene. Under the same conditions, however, ajoene (100 μM) produced a strong inhibition of the thrombin-induced release of dense body and α-granule constituents. Electron spin resonance studies of the effect of ajoene on some physico-chemical properties of the platelet plasma membrane (intact platelets), as well as on artificial lipid membranes, indicated that ajoene increased mobility of the fatty acid spin label 16 nitroxide stearate. This suggests the existence of a decreased microviscosity of the most internal region within the lipid bilayer membrane, without affecting the outer hydrophilic moieties of the bilayer. As a whole, these results suggest that the effect of ajoene on the release reaction must be, in part, due to physical modification of the bilayer, which impairs the fusion of the granules and plasma membrane, a prerequisite for exocytosis.

Garlic has an age-old reputation of possessing a beneficial effect on the circulatory system through improvement of blood fluidity. In 1983, Apitz-Castro *et al.* described anti-platelet properties of a compound derived from garlic [1], which was later named ajoene (*ajo* is garlic in Spanish), characterized as the E,Z-4,5,9-trithiadodeca-1,6,11-triene 9-oxide, and shown to be obtained by rearrangement and *S*-thioallylation of allicin [2, 3]. The anti-platelet action of ajoene is characterised by inhibition of the aggregation induced by all the known inducers *in vitro* and *in vivo*, in a wide variety of animal species (for a review see Ref. 4). Moreover, ajoene prevents the thrombocytopenia induced in dogs by the use of extracorporeal circulation [5]. Inhibition of platelet aggregation by ajoene seems to be causally related to inhibition of fibrinogen binding to the platelet surface, which is a prerequisite for platelet–platelet bridging [6]. Although inhibition of the release reaction has been also observed, no inhibitory effect of ajoene on arachidonic acid metabolism or agonist-induced protein phosphorylation has been demonstrated [1, 7]. At present, the mechanism(s) by which ajoene inhibits the release reaction is far from clear. In order to further define the mode of action of ajoene, we have investigated its effects on phosphoinositide metabolism as well as the physico-chemical properties of the plasma membrane. Our results indicate that ajoene induced modifications of the structural properties of the plasma membrane which may prevent the release of granule constituents.

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MATERIALS AND METHODS

Materials

Ajoene was synthetized and dissolved in ethanol (as in Ref. 1) at a concentration allowing addition of less than 1% of ethanol to platelets. ^{14}C-Serotonin and ^{32}P-orthophosphate were from ORIS (CEA, Saclay, France), metrizamide from Nyegaard (Oslo, Norway), spin labels from Molecular Probe (Eugene, OR). All other chemicals were of purest grade available and were obtained commercially.

Platelet preparation

Platelets were prepared as previously reported [8], using ACD-C as anticoagulant. Briefly, platelets in plasma were labelled either with ^{14}C-serotonin or ^{32}P-orthophosphate, for 90 min at 37°, and separated from excess label through a metrizamide gradient. The platelets thus obtained were adjusted to 3–5 × 10^8 per ml in HEPES 10 mM buffer (pH 7.4), containing 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$ and 10 mM glucose and prewarmed at 37°. Stimulation was performed in the aggregometer and the reactions stopped either by transfer in 1/5 vol. of ice-cold 0.1 M EDTA followed by immediate centrifugation for ^{14}C-labelled

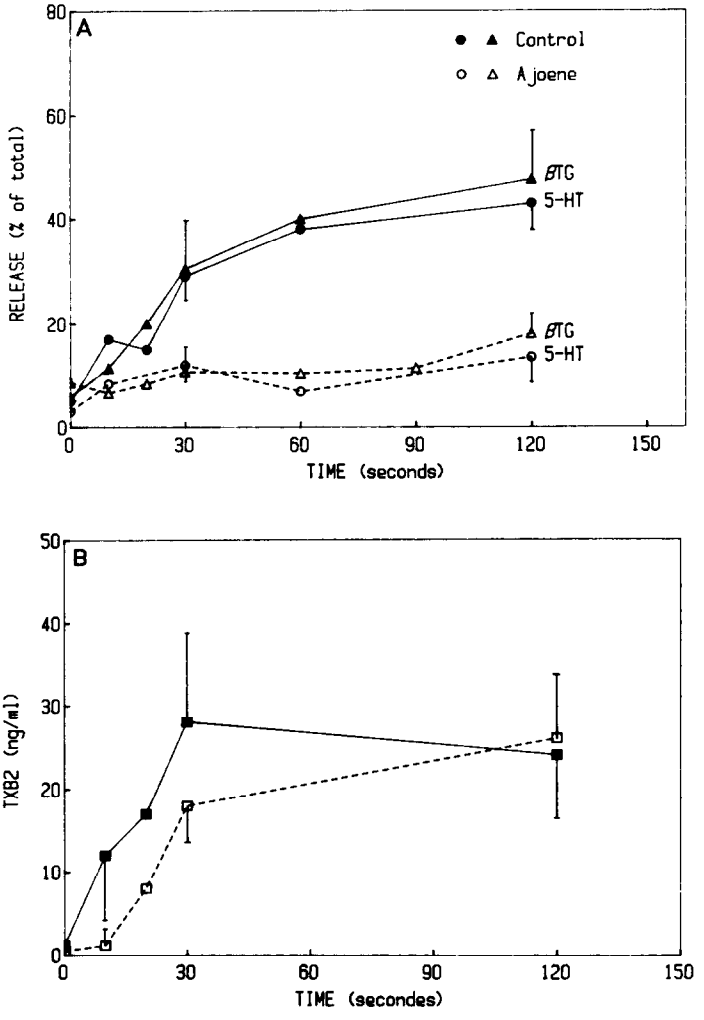

Fig. 1. Inhibitory effect of ajoene on thrombin-induced release. Platelets (3×10^8 plat/ml) were stimulated with 0.05 U/ml thrombin. Ajoene (100 μM) was added 1 min before thrombin. (A) Release from dense bodies (5-HT = ^{14}C-5 hydroxytryptamine or serotonin) and from α-granules (βTG = β-thromboglobulin). (B) Thromboxanes B_2 synthesis. Full symbols, controls; open symbols, in presence of ajoene. (Bars = SD). Control values are the mean of results obtained from experiments performed on 7–10 different donors. Results in presence of ajoene are the mean of three different experiments.

samples, or by addition of 3.75 vol. of chloroform/methanol/12 N HCl/0.1 M EDTA (20:40:1:2 v/v) for ^{32}P samples. Aggregation and release induced by thrombin (0.05 U/ml) were simultaneously measured on the ^{14}C-labelled samples, while phospholipids and protein phosphorylation were analysed on the ^{32}P-labelled platelets, after partition with chloroform.

Aggregation, release and thromboxane formation

Supernatants from ^{14}C-labelled samples were analysed for ^{14}C-serotonin and β-thromboglobulin, using respectively liquid scintillation and a commercial radioimmunoassay kit (Amersham) as previously described [9]. Thromboxane B_2 (TXB_2)* determinations were performed on an aliquot of ^{32}P-labelled platelets in the organic mixture before the phase partition, by enzyme immunoassay using acetylcholinesterase-TXB_2 as label [10].

Phosphoinositide metabolism and protein phosphorylations. The ^{32}P-labelled samples were partitioned by addition of 1/25 vol. each of chloroform and distilled water. A clear biphasic system was obtained by centrifugation.

The lower chloroform phase was processed according to Jolles [11]. Inositol phospholipids and phosphatidic acid were separated by means of one-dimensional thin-layer chromatography on silica plates. After being visualized by autoradiography, phospholipids were recovered by scraping the corresponding spots and their radioactivity counted by liquid scintillation.

The proteins concentrated at the interface of the two phases were solubilised in a Laemmli buffer and after reduction with mercaptoethanol, separated by means of polyacrylamide gel electrophoresis on a

* Abbreviations used: ESR, electron spin resonance; TXB_2, thromboxanes B_2; PC, phosphatidylcholine; PA, phosphatidic acid; PIP_2, phosphatidylinositol 4,5,bis-phosphate.

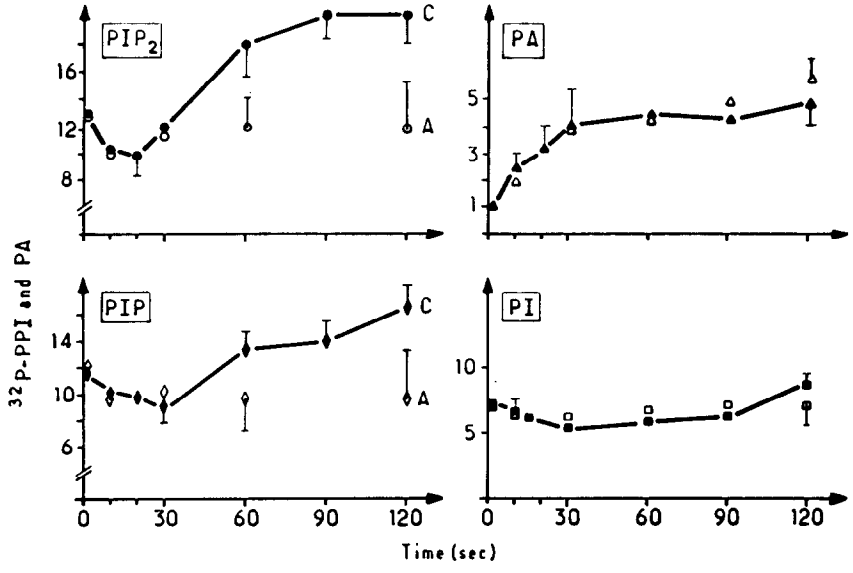

Fig. 2. Time-course of ^{32}P-PPI and ^{32}-PA variations during thrombin (0.05 U/ml) activation, in the absence (full symbols) or presence (open symbols) of ajoene (100 μM). Results are expressed as the ratio of the radioactivity of each phospholipid versus that of ^{32}-PA at time zero. Results in presence of ajoene are the mean of 3–5 different experiments, and control values were obtained as in Fig. 1 (bars = SEM).

13% gel. After staining with Coomassie Blue, destaining and drying, the gels were put in contact with a Hyper-film Beta-max (Amersham), and the autoradiogram scanned with an Ultrascan LKB.

Physical study

Electron spin resonance parameters. Two oxazolidine-nitroxide derivatives of stearic acid were added to membranes as spin labels: 5 and 16 nitroxide stearic acids (5NS, 16NS). The former probes the membrane near the phospholipid polar heads on the fifth carbon atom, the latter in its hydrophobic core on the sixteenth carbon atom. Electron spin resonance (ESR) spectra were recorded as a function of temperature between 1 and 40° with a Varian E 9 spectrometer and analysed with a Hewlett–Packard HP 9825T computer connected to a 9874 A digitizer.

The parameters measured were the outer hyperfine splitting constant (2T//) for 5NS spectra, and the apparent label rotational frequency (νc) for 16 NS label. The 2T// is related to the molecular organization surrounding the probe and accounts for the order parameter. If 2T// increases, the order increases at this level of the membrane, i.e. at the outer hydrophilic moieties of the bilayer. The 16NS spectra parameter νc describes the movements of the probe. If the membrane internal microviscosity decreases, the probe can move more freely and νc increases. Thus νc gives indications about the molecular cohesion in the inner part of the lipid bilayer.

Platelets. Platelets were concentrated through a metrizamide gradient as above, except that the final concentration was adjusted to 3.10^9 cells/ml. This represents a high cell concentration as compared to that used for physiological or biochemical responses but is necessary for an accurate study in ESR. Platelets were immediately used for ESR experiments as previously described [12]. With hand-shaking, 2 μl of a 10 mM solution of spin label in dimethylsulfoxyde were added to 250 μl of platelet suspension. In the case of 16 NS, platelets were washed once more in order to eliminate free label. Labelled platelets were drawn up into glass siliconized capillaries (i.d. 0.7 mm), four capillaries were introduced in a quartz holder tube, and spectra recorded.

Artificial lipidic membranes. Large unilamellar vesicles were prepared as previously described [13]. A mixture of 90% of L-alpha-phosphatidylcholine (PC) prepared from egg-yolk, 10% phosphatidic acid (PA) and cholesterol in chloroformic solution was evaporated in a Rotavapor, resuspended in ether and mixed to 400 mM sodium phosphate containing 1 mM EDTA (pH 5.5). The mixture was sonicated under nitrogen (3 × 2 min) and the solvent was evaporated with 40 rotations/min in order to constitute the vesicular phase. After addition of 400 mM sodium sulfate, three successive filtrations were performed respectively on 0.8-, 0.4-, and 0.2-μm diameter filters (Nucleopore Corporation), respectively. Final lipid concentrations were 18 mM. Some of the vesicles were enriched in cholesterol by using a mixture of 80% PC, 10% PA and 10% cholesterol. Spin labels were added in the same conditions as for platelets.

RESULTS

Inhibition of the release reaction

As shown in Fig. 1A, ajoene (100 μM, 1 min of incubation) inhibited thrombin-induced (0.05 U/ml) release reaction by washed platelets by over 60%. As can be seen, both ^{14}C-serotonin and β-thromboglobulin release were inhibited. Addition of ajoene immediately before thrombin also resulted in 50%

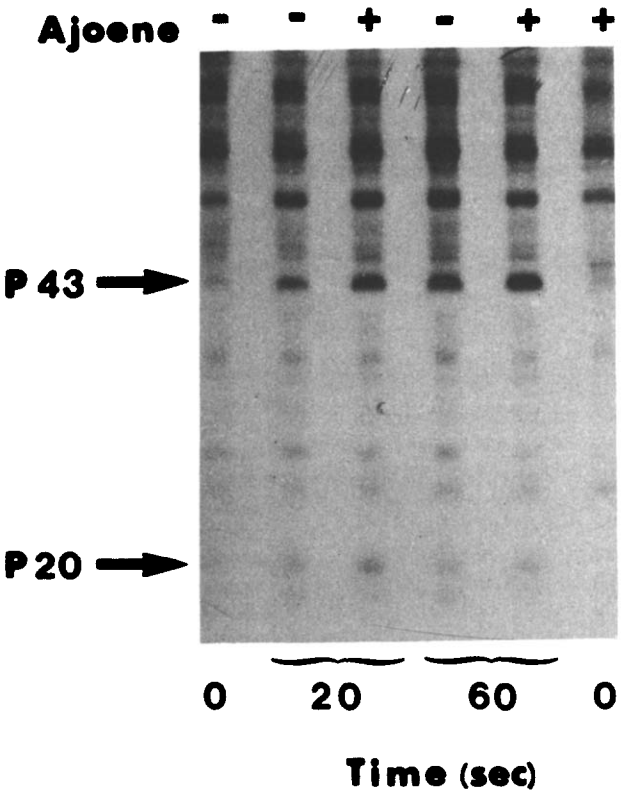

Fig. 3. Autoradiograms of P20 and P43 protein phosphorylations during thrombin (0.05 U/ml)-induced activation in absence or presence of ajoene (100 μM).

and 63% inhibition of ^{14}C-serotonin, at 10 and 60 sec respectively, after addition of the agonist. During the same time period, ajoene had a minimal effect on thromboxane formation (Fig. 1B) considering the great variations observed from one donor to the other using this thrombin concentration (0.05 U/ml).

As previously reported [1], the extent of the inhibition of the release reaction was markedly dependent on the concentration of ajoene, attaining 40, 50 and 80% with 33, 66 and 132 μM of ajoene respectively (not shown).

Effect of ajoene on inositide metabolism

The effect of ajoene (100 μM) on the time course of the hydrolysis and resynthesis of phosphatidylinositol 4,5,bisphosphate (PIP_2), phosphatidylinositol 4,phosphate (PIP) and phosphatidylinositol (PI) is shown in Fig. 2. Control experiments show that thrombin stimulation (0.05 U/ml) produces a rapid and transient decrease of PIP_2, PIP and PI. These decreases were followed by a slower resynthesis of the inositides and after about 2 min, the values for PIP_2 and PIP were higher than the prestimulation values by about 30%. Ajoene did not affect the initial, rapid, hydrolysis of any of the phosphoinositides analysed. However, the resynthesis phase seemed to be affected since the values attained in the presence of ajoene reached at most the pre-stimulation level. Unstimulated platelets do not contain any ^{32}-phosphatidic acid under labeling experiments. This is formed in the first seconds following addition of thrombin. As shown in Fig. 2, ajoene also did not affect the rapid thrombin-induced synthesis of ^{32}P-phosphatidic acid (PA).

Effect of ajoene on protein phosphorylation

As shown in Fig. 3, incubation of platelets with ajoene did not affect the thrombin-induced phosphorylation of the myosin light chain (P20) or of the protein of 43 kD (P43).

Membrane structural properties

Intact platelets. Two spin labels, 5NS and 16 NS, were used to study the effect of ajoene on platelet membrane structural properties. As shown in Fig. 4, incubation of intact platelets with ajoene (even at 320 μM) did not induce significant change in the outer hyperfine splitting constant 2T// of the 5 NS spin label, indicating no effect of ajoene on the organization of the outer part of the membrane.

Under the same conditions, ajoene (even at 80 μM), induced an increase of the correlation rotation frequency of the 16 NS spin label νc in all the range of temperatures explored (Fig. 5). The slope of the curve $\ln \nu c = f(1/T°K)$ has the dimension of an energy of activation: it corresponds to the thermal susceptibility of the membrane [12], and represents the loss of molecular cohesion when the temperature increases. The increase of this slope in presence of ajoene indicates that the molecular cohesion in the hydrophobic core of the membrane is looser and thus more susceptible to the increase in temperature.

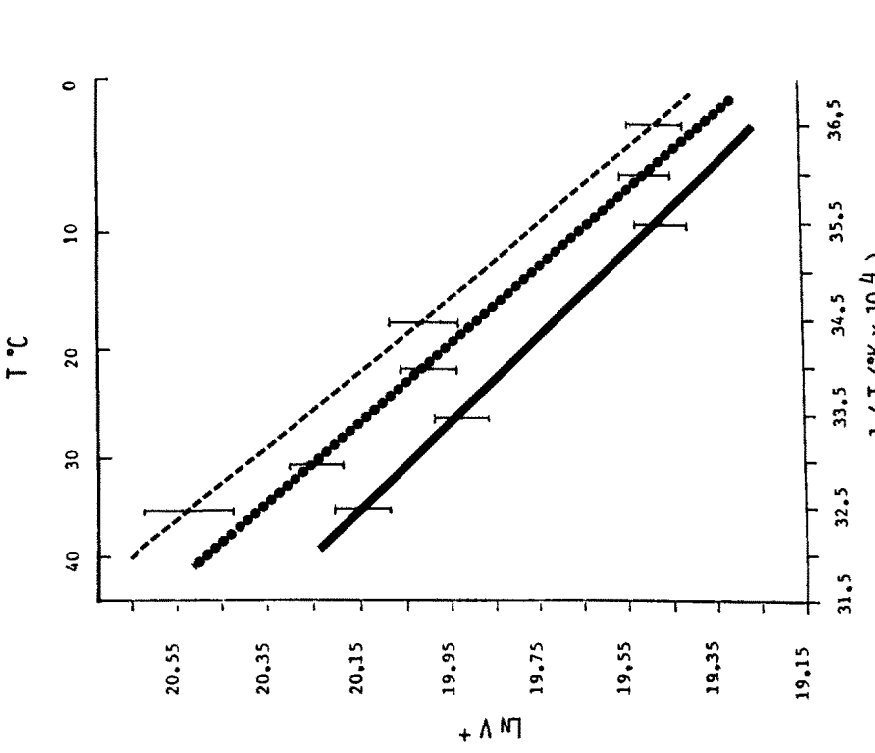

Fig. 5. Arrhenius plot of the variation of the correlation rotation frequency ν_c of the 16 NS spin-labelled platelets, in the absence (—), or in the presence of 160 μM (●●●) or 320 μM (– – –) ajoene. Results are mean of three different experiments (bars = SD).

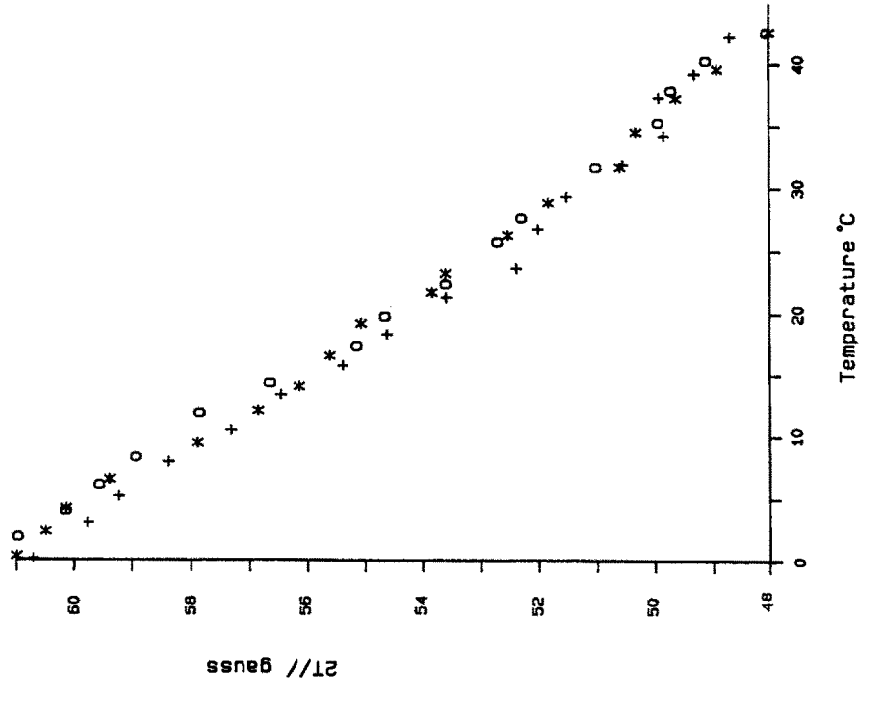

Fig. 4. Temperature dependence of the outer hyperfine splitting constant 2T// of the 5 NS spin-labelled human platelets, in the absence (○) or in the presence of 160 μM (*) or 320 μM (+) ajoene. Results are the mean of three different experiments.

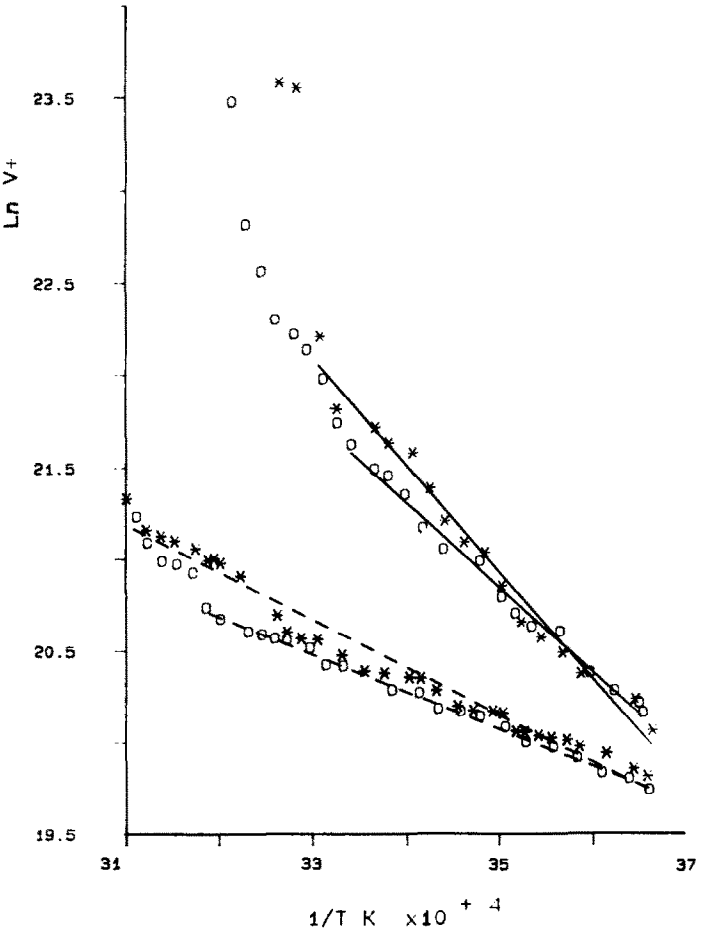

Fig. 6. Arrhenius plot of the correlation rotation frequency νc of the 16 NS spin-labelled artificial vesicles enriched (dashed lines) or not (plain lines) in cholesterol, and in the absence (○) or presence (*) of 160 μM ajoene.

Effect of ajoene on artificial membranes. In order to further investigate the ajoene-induced hydrophobic core modifications probed with the 16 NS label, we have studied the ajoene effect on large unilamellar vesicles. These vesicles show an increase in fluidity above 12° as shown by the increase of the slope of the curve ln $\nu c = f(1/T°K)$. Addition of ajoene to these vesicles induced an increase of the mobility of the 16 NS spin label in all the range of temperatures explored, with a complete destabilization of the membrane for temperatures over 30° (Fig. 6).

In cholesterol-enriched vesicles, as a consequence of the stabilizing effect of cholesterol, the thermal susceptibility is decreased. Under these conditions, although the molecular cohesion is stronger, the presence of ajoene also induced an increase of the 16 NS spin label mobility in all the range of temperatures explored. Furthermore, the relative increase in the thermal susceptibility was similar to that measured in vesicles without cholesterol.

DISCUSSION

Ajoene has been shown to be an inhibitor of platelet aggregation and release *in vitro* and *in vivo* [1, 4, 5]. The inhibitory effect of ajoene on the consolidation of irreversible platelet–platelet interaction seems to be related to an impairment of the platelet ability to interact with fibrinogen [6]. Our results clearly demonstrate that in addition, ajoene inhibits the release of the constituents of dense bodies and α-granules induced by thrombin. The latter effect does not seem to be causally related to the inhibition of fibrinogen binding, since compounds such as RGDS [14–16] or trigramin [17], which are competitive inhibitors of fibrinogen binding to platlets, do not have any effect on the release reaction.

Stimulation of platelets by agonists such as thrombin is mediated by a receptor-dependent activation of the hydrolysis of membrane polyphosphoinositides [18], and release of granule contents seems to be in part related to a protein kinase C-mediated phosphorylation of a 43kD protein, P43 [19]. It is conceivable that inhibition of any of these early steps of platelet activation could be responsible for the effect of ajoene on the release reaction. Our results show that ajoene does not affect the thrombin-induced activation of membrane-bound phospholipase C, since hydrolysis of PIP_2, PIP and PI, and phosphatidic acid synthesis in the presence of ajoene,

were not different from control platelets. At present we do not have a clear explanation for the moderate inhibition observed on the resynthesis of PIP_2 and PIP in ajoene-treated platelets. Our results further indicate that ajoene does not inhibit the thrombin-mediated activation of protein kinase C or the calcium calmodulin-dependent protein kinase, since normal patterns of phosphorylation of the protein P43 and the myosin light chain P20 were obtained in the presence of ajoene. Thus the calcium mobilization does not seem to be affected, as is also suggested by the normal production of thromboxanes (which is mainly due to the activity of a phospholipase A_2) by ajoene-treated platelets.

That the transducing system involving phospholipase C was not affected was confirmed by the fact that ajoene did not modify the outer hyperfine splitting constant of the 5 NS spin label, i.e. the phospholipid organization near their polar heads. Indeed, we have previously related the thrombin-induced phospholipid disorganization to the PIP_2 breakdown [20]. The results obtained with the 16 NS spin label strongly suggest that ajoene induces a dramatic decrease in the molecular cohesion (microviscosity) of the inner part of the membrane bilayer. This is emphasized by the great increase of the 16 NS label mobility in cholesterol-enriched artificial membranes, since cholesterol is well known for its membrane stabilizing effect. A general, nonspecific alteration of the membrane does not fit with the highly selective effect of the compound on the fibrinogen receptor, without impairment of other membrane processes such as agonist interactions, shape change [1, 7], phosphoinositide turnover, etc. These suggest that ajoene might be acting on a specific domain of the plasma membrane.

Two mechanisms for the release of granule constituents in platelets have been proposed; a rapid one by direct lipophilic exocytosis [21, 22] and a slower process which involves centralization of the granules within a bundle of microtubules [23, 24]. Although ajoene does not affect microtubule reorganization, the hypothesis that ajoene could interact with cytoskeletal components cannot be excluded. Since no clear-cut sequential scheme between protein phosphorylation and release has been established, ajoene may well affect an as yet undefined step occurring between protein phosphorylation and release. Taken as a whole, our present results strongly suggest that the effect of ajoene on the release reaction must be due, in part, to the alteration of the microviscosity in the inner part of the plasma membrane. Such modification may have an important effect on the process of fusion of the granule membrane with the plasma membrane, a prerequisite for exocytosis.

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