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THE MOLECULAR BASIS OF THE ANTIPLATELET ACTION OF AJOENE: DIRECT INTERACTION WITH THE FIBRINOGEN RECEPTOR

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SUMMARY: Ajoene, the major antiplatelet compound derived from garlic inhibits the fibrinogen-supported aggregation of washed human platelets (ID_{50} = 13 uM) and, inhibits binding of ^{125}I-fibrinogen to ADP-stimulated platelets (ID_{50} = 0.8 uM). In both cases, the inhibition is of the mixed non-competitive type. Furthermore, fibrinogen-induced aggregation of chymotrypsin-treated platelets is also inhibited by ajoene in a dose-dependent manner (ID_{50} = 2.3 uM). Other membrane receptors such as ADP or epinephrine receptors are not affected by ajoene. Ajoene strongly quenches the intrinsic fluorescence emission of purified glycoproteins IIb-IIIa (ID_{50} = 10 uM). These results indicate that the antiaggregatory effect of ajoene is causally related to its direct interaction with the putative fibrinogen receptor.

Ajoene (E,Z)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide, a potent antiplatelet compound derived from garlic (1,2) inhibits platelet aggregation induced in vitro by ADP, collagen, epinephrine, thrombin, ionophore A-23187, platelet aggregating factor, arachidonic acid (1) and the endoperoxide analog U-46619 (unpublished). Inhibition of aggregation by ajoene does not affect shape change, arachidonate metabolism or cAMP levels (1). It acts synergistically with antiaggregatory compounds such as prostacyclin, indomethacine or dipyridamole (3). These observations suggest that the inhibitory action of ajoene on platelet activation must related to blockade by ajoene of some common step, in the chain of reactions leading to platelet aggregation and that this step must be shared by all known agonists of platelet activation. Regardless of the agonist, the platelet fibrinogen receptor plays this pivotal role in platelet aggregation (4). Here we report that ajoene inhibits fibrinogen binding to ADP- and chymotrypsin-treated platelets (CTP), and shows high affinity interaction with purified fibrinogen receptors from human platelets.

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METHODS

The protocols used in this study are described elsewhere (1,3) or given in the figure caption. Epinephrine receptors were studied as described by Macfarlane et al. (4), using ^{3}H-Rauwolscine as antagonist. Glycoproteins IIb-IIIa were purified as described by Newman et al. (5).

RESULTS AND DISCUSSION

The effect of ajoene on the fibrinogen-supported aggregation of gel filtered platelets (GFP) activated by ADP is shown in Fig. 1A. This effect of ajoene, as have been found with other agonists (1,3), shows a sigmoidal dependence on the concentration of the inhibitor, with ID_{50} = 13 uM and complete inhibition obtained above 20 uM ajoene. Graphical analysis of the data (Fig. 1A, insert) yields patterns compatible with a mixed non-competitive inhibition.

As shown in Fig. 1B, incubation of GFP with ajoene inhibits the ADP-induced binding of fibrinogen. As the concentration of fibrinogen increases, the effect of ajoene on binding is more pronounced, reaching about 95% inhibition at a fibrinogen concentration of 2 uM (data not included in the graph). It has been shown that saturation of fibrinogen receptors at high fibrinogen concentrations (> 14 uM) leads to decreased platelet aggregation (6,7). A possible explanation for these observations is the existence of negative cooperative interactions among platelet fibrinogen receptors (8). Our data, suggests that these negative interactions become accentuated in the presence of ajoene, which somehow modulates the apparent down regulation of the receptors by fibrinogen. This working hipothesis is consistent with the apparently non-competitive inhibition of aggregation and fibrinogen binding (inserts, Fig. 1A and B). Inhibition of binding of fibrinogen to intact platelets shows a hyperbolic dependence on the concentration of ajoene (Fig. 1C, curve 1). More than 98% inhibition is observed at about 5 uM ajoene, and ID_{50} is about 0.8 uM. This suggests that the effect of ajoene on binding of fibrinogen is causally related to the effect of ajoene on aggregation of platelets induced by the various agonists and supported by fibrinogen. This idea is further supported by the fact that inhibition of aggregation by ajoene shows an inverse dependence on the number of platelets in the sample. The fact that ID_{50} values for the inhibition of binding and aggregation differ by a factor of about 10 suggests that the functional coupling between the various processes, ranging from exposure of the fibrinogen receptor to the formation of irreversible aggregates, may not involve a simple direct progression of events from the initial ligand-receptor interaction to the final response. Fig. 1C (curve 2), shows that once bound, fibrinogen is not displaced by ajoene, even at 100 M concentration. This is in contrast with the observation that ajoene does disaggregate platelets that have been challenged with ADP or collagen, in PRP (1). Unfortunately, very little is known about the factors that modulate

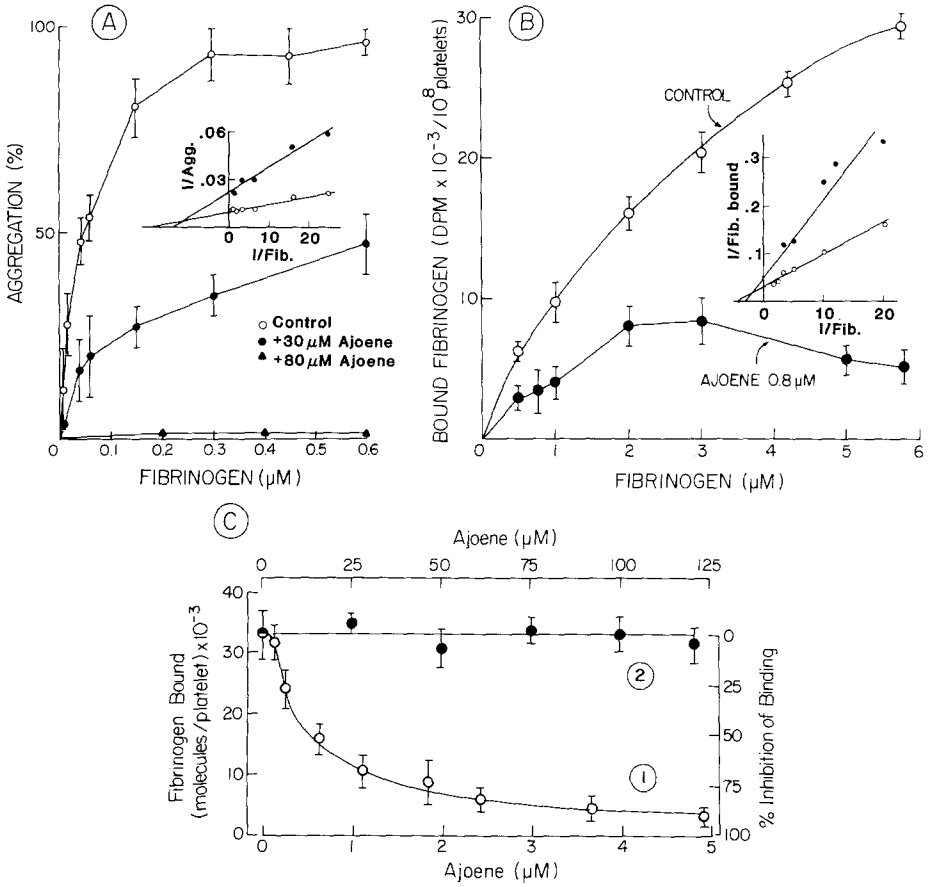

FIGURE 1. A) Effect of ajoene on fibrinogen-supported aggregation of gel filtered platelets (GFP) obtained as described by Lages et al (14). Aggregation induced by 10 uM ADP, at 37° C, in the presence of 1 mM calcium. Synthetic ajoene (2) was added 90 seconds before activation of the platelets with ADP. Solvent (ethanol) was used in the control GFP. Data represents mean ± s.d. of six independent experiments. Ordinate represents extent of aggregation. Insert, double-reciprocal plot of the data. B) Inhibition by ajoene of fibrinogen binding to ADP stimulated platelets. Human fibrinogen was radio-iodinated by the Iodogen method (15) (specific activity = 2 mCi/umole). GFP suspended in Hepes-Tyrode, pH 7,4 (1) was mixed with ^{125}I-fibrinogen to give a final fibrinogen concentration = 0.3 uM and calcium concentration = 1 mM. Platelet activation was initiated with 10 uM ADP. After 30 min. incubation at 25° C, the platelets were sedimented through a layer of silicone. Ajoene was added 90 seconds before the agonist. Nonspecific binding was measured in the presence of 20 mM EGTA. Data represents mean ± s.d. of three independent experiments. Insert, double-reciprocal plot of the data. C) Concentration dependence of the effect of ajoene on binding of fibrinogen to GFP. Experimental conditions as described in B. Curve 1, ajoene added 90 seconds before addition of the agonist (ADP = 10 uM). Curve 2, ajoene added 30 minutes after addition of ADP. Data represents mean ± s.d. of five independent experiments.

functional expression of fibrinogen receptors in platelets, however, the apparent discrepancy mentioned above may be simply related to the experimental conditions used for binding (30 min. incubation before addition of ajoene)(9). Chymotrypsin-treated platelets bind fibrinogen and aggregate when stirred in the presence of fibrinogen, without addition of any other agonist (7).

Although the mechanism of the exposure of fibrinogen receptors by chymotrypsin is still unclear, it appears that an intact IIb-IIIa complex is necessary for fibrinogen binding to CTP. Ajoene inhibits, in a dose-dependent manner (ID_{50} = 2,3 uM), the fibrinogen-supported aggregation of platelets whose fibrinogen receptors have been "exposed" by chymotrypsin following the procedure described by Kornecki et al (10). Since aggregation of CTP does not require of a metabolically active cell (11) and is not blocked by compounds that elevate intraplatelet levels of cAMP (12), the inhibition by ajoene must be interpreted as a direct effect on the aggregation process mediated by functional fibrinogen receptors. This conclusion is also consistent with the observation that the effect of ajoene does not involve contractile or motile functions arising from cytoplasmic structures because thrombin-induced phosphorylation of contractile proteins such as myosin light chain or of the secretion related peptide P47 is not inhibited by ajoene (data not shown here).

A direct effect of ajoene on the isolated receptor for fibrinogen could also be demonstrated. The functional fibrinogen receptor in platelets have been identified with a calcium-dependent complex of membrane glycoproteins IIb and IIIa (see Ref. 4 for a review on the subject). Under appropriate conditions, the GP IIb-IIIa complex can be isolated from platelet plasma membranes in rather pure and functionally active state (5,13). We made use of the intrinsic fluorescence of tryptophan from the isolated IIb-IIIa complex to monitor its interaction with ajoene. Glycoprotein IIb-IIIa complex exhibits a large fluorescence emission maximum at 328 nm (excitation at 290 nm). The emission intensity decreases in the presence of ajoene. Addition of 10 mM EGTA does not affect the position of the maximum or the quantum yield of fluorescence, and does not influence the effect of ajoene. The concentration dependence for the quenching fits a Michaelis-Menten type of hyperbola; half of the total intensity is quenched by about 10 uM ajoene and more than 95% of the fluorescence intensity is quenched at about 100 uM ajoene. These observations suggest that ajoene binds with high affinity, and that an intact IIb-IIIa complex is not a requisite for the interaction. The effect of ajoene on the fluorescence emission can be reversed by high concentrations of EGTA (30 mM) suggesting a non-covalent type of interaction.

The kinetic patterns obtained from both, the aggregation and binding experiments, which suggest a non competitive type of interaction, further support the idea that ajoene interacts with the IIb-IIIa complex (or part of it) at a site(s) which is different from the fibrinogen binding site(s).

The interaction of ajoene with the fibrinogen receptor in the membrane seems to be also highly selective. Elsewhere we have shown that ajoene inhibits the action of the various inducers of aggregation without any noticeable effect on

the shape change (1). This implies that the interaction of inducers with their primary receptors on the platelet surface is not blocked by ajoene. This is directly substantiated at least for the epinephrine receptors. Ajoene (10 uM) does not modify the affinity (2.7 $\pm$ .8 uM) nor the number (320 $\pm$ 20 per platelet) of epinephrine receptors in intact, gel filtered platelets, measured as described by Macfarlane et al (5), using ^{3}H-Rauwolscine as antagonist (n = 5, data not shown here).

The results reported here represent the first direct demonstration of a specific interaction of an antiplatelet compound, different from a monoclonal antibody, with the fibrinogen receptors of the platelet surface. Presence of a high affinity binding site for a small molecule like ajoene on the fibrinogen receptor raises the possibility that this pivotal step in the sequence of platelet reactions may be regulated in vivo by a yet unidentified factor that shares the binding site for ajoene. Furthermore, our results lend a molecular support to the antiaggregatory effect of ajoene on human platelets and accentuate the importance of the fibrinogen receptors in the design of selective, general antiplatelet agents.

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