Vol. 153, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1988 Pages 479-486

AJOENE INHIBITION OF PLATELET AGGREGATION : POSSIBLE MEDIATION BY A HEMOPROTEIN

Moideen P. Jamaluddin, Lissy K. Krishnan & Ancy Thomas

Thrombosis Research Unit, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Trivandrum-695012, INDIA

Received April 29, 1988

SUMMARY. Ajoene, an organosulfur compound derived from garlic, was found by spectral measurements, to interact, cooperatively, with a purified hemoprotein implicated, previously, in platelet activation. It modified the binding interactions of the protein with ligands, deemed to be physiologically relevant as effectors. The characteristics of the modifications were found to parallel those of ajoene induced modifications of agonist-induced aggregation kinetics of gel-filtered calf platelets.

INTRODUCTION. Ajoene, (E,Z)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide, derived from garlic, irreversibly inhibits platelet aggregation without affecting shape-change, metabolism of endogenous arachidonate, cAMP levels, and protein phosphorylation (1-4). Recent evidence suggests its mechanism of action to be by direct interaction with the fibrinogen receptor of platelets (4). This mechanism, however, fails to explain the apparent cooperativity of its inhibitory action (4). Therefore an additional or alternative site of action is suggested. We have implicated ligand-induced conformational change of a dimeric hemoprotein in platelet activation (5,6). Here we report on a parallelism found between the modifying effects of ajoene on ligand binding to this protein and the modifications it effected on the kinetics of agonist-induced aggregation of calf platelets.

MATERIALS AND METHODS. Ajoene was purified from freshly peeled, crushed, garlic employing an adaptation of published procedures (2). The purified compound was kept dissolved in 0.13 M NaCl, at -30°C. H_2O_2 was a product of Glaxo (India). Other chemicals were from commercial sources given before (5,7-9). Gelfiltered platelets (GFP) were prepared as described (7-9) except for the following modifications: acid-citrate-dextrose solution replaced trisodium citrate as anticoagulant (1.5 ml per 8.5 ml of blood)

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and calcium was omitted from the elution buffer but added to the GFP 5 min before aggregation studies (with ADP only). Purification of hemoprotein was described (5). Platelet aggregation was assayed as in (7). Polyacrylamide gel electrophoresis (PAGE) was performed at 4°C and at 5 mA/tube, in 10% cylindrical gels using 0.1 M sodium phosphate buffer, pH 7.2. Gels were stained with Coomassie brilliant blue R. All values reported are mean ± SE of three independent experiments.

RESULTS AND DISCUSSION. The compound purified from garlic showed IR, UV and NMR spectral properties similar to those reported for ajoene (2). As found earlier for human platelets by conventional aggregometry (1), ajoene inhibited ADP-induced aggregation of calf GFP assayed by the spectrophotometric method, dose-dependently, without reducing shape-change (Fig.1A).

According to the sequential shape-change and interaction model of platelet aggregation, kinetics of platelet aggregation and inhibition may be treated similarly to enzyme kinetics (10). So the ajoene inhibition data were plotted on double-reciprocal and Hill coordinates (Fig.1B). The double-reciprocal plot was curved concave down. The Hill plot was biphasic with $h<1$ (0.3±0.1), or apparent negative cooperativity, at low ajoene concentrations and $h>1$ (3.2±0.2), or apparent positive cooperativity, at higher ajoene concentrations. The half-maximal inhibitory concentration ($I_{0.5}$) was 15±3 μM.

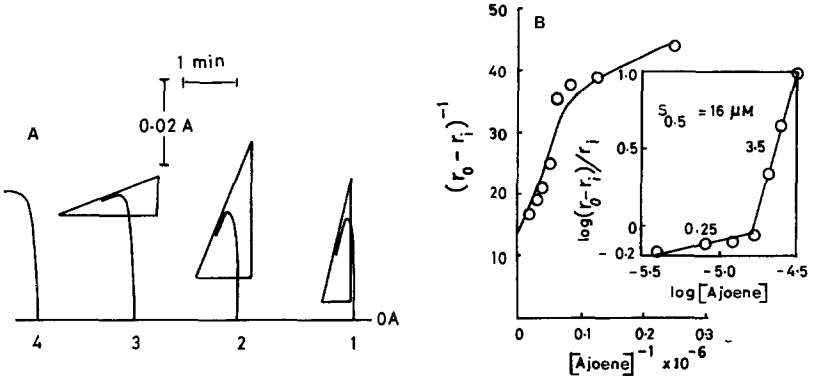


Fig.1A. Pattern of inhibition of ADP-induced platelet aggregation by ajoene, assayed spectrophotometrically. The absorbance scale and chart-speed employed are indicated at the top. The manner of measuring initial rate (r_o), is shown. Tracings 1-4, were obtained on adding ADP (13.6 μM) to the platelets preincubated for 1 min with ajoene at the final concentrations (μM) of 0, 16, 32 and 64, respectively. B. Typical double-reciprocal and Hill plots of the kinetics of inhibition of ADP-induced aggregation by various concentrations of ajoene. r_o and r_i are, respectively, rates in the absence and in the presence of ajoene.

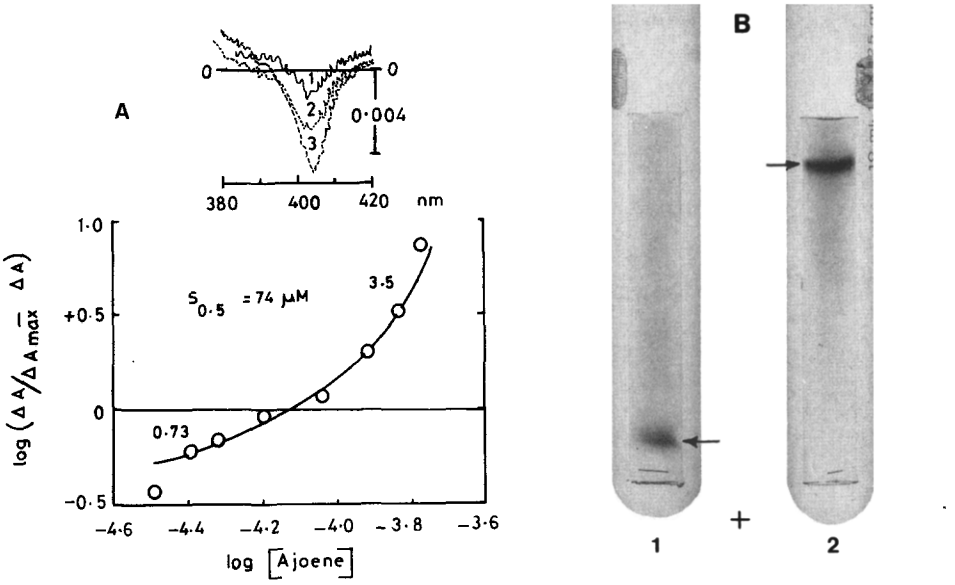


Fig.2A. Typical difference spectra obtained on treating the purified hemoprotein with increasing concentrations of ajoene, and the Hill plot of the data. The protein (1 ml, 0.9 μM) in 0.05 M sodium phosphate buffer, pH 7.4, was employed. Ajoene was added to the sample cuvette and an equal volume of reagent diluent to the reference cuvette at 28°C. Spectra numbered 1,2, and 3 (top) were those obtained after adding 48, 98 and 170 μM ajoene. Δ A's of the Hill plot (bottom) were the $(A_{420}-A_{405})$ values. B. Effect of ajoene on the electrophoretic mobility of the hemoprotein in polyacrylamide gels. The protein (13 μg) treated with reagent-diluent or ajoene (120 μM, final concentration) for 5 min at 30°C was subjected to electrophoresis towards the anode at the bottom. Arrows show protein band in the gel with the control protein (1) or ajoene-treated protein (2). Experimental conditions were as given in the text.

The interaction of ajoene with the purified hemoprotein followed by difference-spectral measurements exhibited the concentration-dependence of Fig.2A. The Hill plot of the spectral data revealed similarity to Fig.1B, with $h < 1$ (0.7±0.1) at low ajoene concentrations and $h > 1$ (3.0±0.3) at higher ajoene concentrations but the half-maximal saturation concentration, $S_{0.5}$(60±10 μM), was about 4-fold higher than $I_{0.5}$. This sort of difference between the half-saturation concentrations of binding and biological effect could, however, arise from participation of interactive steps between binding and manifestation of inhibition (11).

Ajoene irreversibly altered the conformation of the protein as shown by a drastic reduction of its electrophoretic mobility (Fig.2B) and changes in its elution pattern in gel-permeation chromatography (not shown). Concomitantly, the lig-

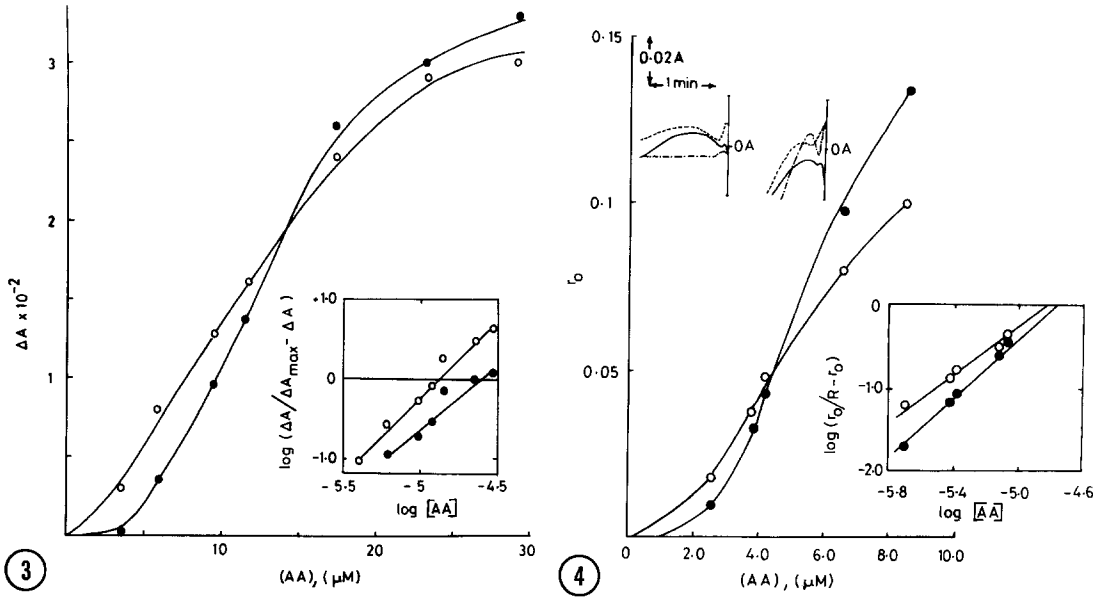


Fig.3. Modification of the binding interaction of arachidonate with the platelet hemoprotein by ajoene. The protein (0.7 μM) treated with ajoene (64 μM) or reagent diluent, for 30 min at 28°C was used for binding studies by difference spectral measurements. Δ A's represent (A_{420}-A_{402}) values at different arachidonate concentrations, employing ajoene - treated protein (●) or diluent-treated protein (○). Hill plots of the data are shown in the inset. Δ A_{max} values for the Hill plots were obtained from double-reciprocal plots (not shown). The $S_{0.5}$ and h values in the absence of ajoene were 13 μM, and 1.9, respectively. The corresponding values in the presence of ajoene were 25 μM, and 1.6, respectively. Data representative of two other experiments with similar results reported.

Fig.4. Modification of the kinetics of arachidonate-induced aggregation of calf GFP by ajoene. Aggregation was assayed as in Fig.1A employing arachidonic acid (in dimethyl sulfoxide). The concentration of dimethyl sulfoxide, 0.5%, v/v, did not affect platelet functions. The changes of r_o values as a function of arachidonate concentration in the absence (○), or in the presence of ajoene (12.5 μM) (●), are shown. The Hill plot of the data are presented in the lower inset. In the upper inset, representative pen response patterns in the absence of ajoene (—) or in the presence of ajoene, (12.5 μM) (---) or (25 μM) (-.-) are shown. The patterns at the left and right represent those obtained with 2-3 and 4-5 μM arachidonate, respectively. The $S_{0.5}$ and h values of the data in the absence of ajoene were 15 μM and 1.5, respectively. The corresponding values in the presence of ajoene were 17 μM and 1.8, respectively.

and-binding properties of the protein changed. With arachidonate as ligand both $S_{0.5}$ and maximum binding increased while h decreased (Fig.3). With H_2O_2, $S_{0.5}$ increased from~10 μM to ~35 μM and h decreased from~2 to~1.3, without change in maximum binding (data not shown), indicating competition at a common site, probably the heme-iron.

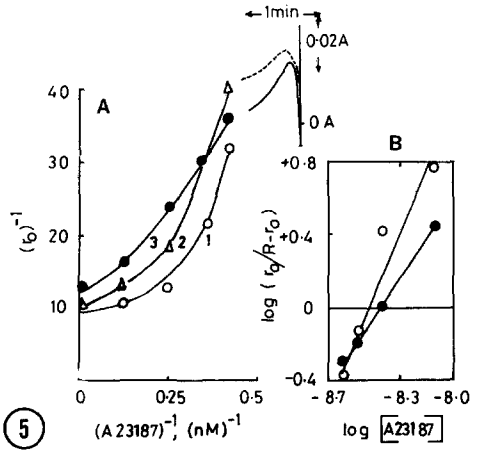
A prediction from the data in Fig.3, that ajoene should inhibit arachidonate-induced aggregation at low agonist concentrat-

ions and activate it at high agonist concentrations, was realized (Fig.4). But inhibition was found only at arachidonate concentrations giving low rates. Then both shape-change and aggregation could be abolished by 25-40 μM ajoene (Fig.4, top inset, left). Contrary to the predictions of Fig.3, however, ajoene did not affect $S_{0.5}$, significantly, and changed $\underline{h}$ in the opposite direction. Aggregation of calf GFP by high arachidonate could be unnatural because it induced only shape-change, no aggregation, in citrated calf platelet-rich plasma (not shown). Therefore we turned to the calcium ionophore, A23187, which acts by way of arachidonate release and metabolism (12).

Immunofluorescence studies using anti-IgG (rabbit) revealed that an antigenically similar protein was present on whole GFP; and anti-IgG, but not nonimmune-IgG, inhibited A23187-induced aggregation (data not shown) confirming the role of this protein in platelet activation by A23187. It seemed reasonable to assume that this protein was accessible to ajoene whose polar nature might prevent its entry into platelets.

The kinetics of A23187-induced aggregation showed a positively cooperative pattern ($\underline{h} > 1$) which ajoene modified by inhibiting rates (Fig.5). No activation was detected. But results with platelets in which conversion of arachidonate to prostaglandin H_2 (PGH_2) was blocked by treatment with acetyl salicylic acid (aspirin) suggested that a net result of inhibition and activation might have been measured; in the presence of ajoene, aspirin-treated platelets gave lower rates (net inhibition) at lower agonist concentrations, and higher rates (net activation) at higher agonist concentrations (Fig.5A, curve 2) compared to control platelets (curve 3). We interpret these results as indicating that ajoene monitored a shift of effector specificity and emphasis from PGH_2/thromboxane A_2 in control platelets, to arachidonate in aspirin-treated platelets.

Among a large number of ligands we have tested so far, as possible effectors on the protein, PGH_2 showed large spectral shifts and high-affinity binding ($S_{0.5}$= 1-2 μM) reaching saturation, cooperatively ($\underline{h}$ = 2), at a ligand: protein molar ratio of 2. PGH_2 was followed by H_2O_2, oleic acid, arachidonic acid and several other unsaturated fatty acids (unpublished results). The latter compounds, apparently, recognized some aspects of the PGH_2 binding site on the protein.



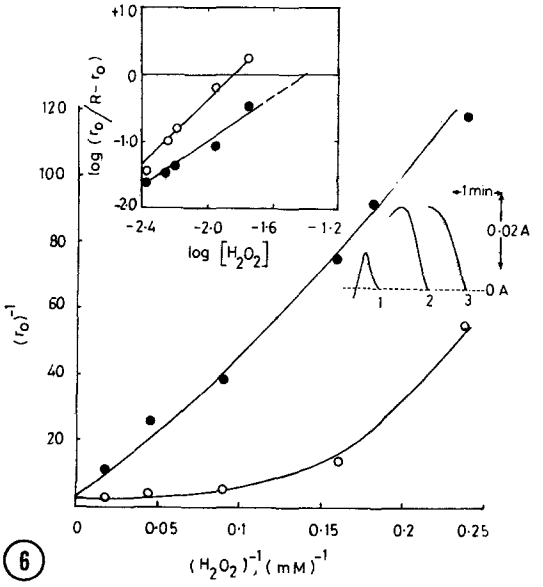


Fig.5. Modification of the kinetics of A23187-induced aggregation of calf GFP by ajoene, reported on double-reciprocal (A) and Hill (B) coordinates. r_o values were measured as in Fig.1A using A23187, dissolved in dimethyl sulfoxide, in the absence (O) or in the presence of (25.5 μM) ajoene (●). The inset shows the pen-response patterns obtained at 3 nM A23187 without ajoene (—) or with ajoene (---). Curve 2 in A, was obtained in the presence of ajoene (12.5 μM) employing platelets (the same batch as that used in curve 3) that were preincubated with 1.5 mM acetylsalicylic acid (aspirin) for 30 min at 30°C. Alternate assays were done in triplicate, with aspirin-treated or untreated platelets. Each experimental point is the average of two or three values which agreed with each other within 10%, or less. The values of $S_{0.5}$ and h in the absence of ajoene were 3.4 nM and 2.4, respectively. r_o values varied with donors but qualitatively similar results were obtained with two other donors.

Fig.6. Modification of the kinetics of H_2O_2-induced aggregation of calf GFP by ajoene reported on double-reciprocal and Hill Coordinates. Data obtained in the absence (O) or in the presence of (20 μM) ajoene (●) are shown. Spectrophotometric pen response patterns obtained with 6 mM H_2O_2 in the absence of ajoene (1) and in the presence of ajoene (20 μM) (2), or (80 μM) (3) are shown in the lower inset. The $S_{0.5}$ and h values in the absence of ajoene were 14 mM and 2.4, respectively. They changed to 40 mM and 1.7, respectively, in the presence of ajoene. One of two similar experimental results reported.

H_2O_2, presumably acting as an analog of PGH_2, induced strong platelet aggregation; and ajoene modified its aggregation kinetics (Fig.6) in a manner that reminded its modification of the binding of H_2O_2 to the protein, decreasing h, and markedly increasing $S_{0.5}$. Furthermore, ajoene abolished H_2O_2-induced aggregation even as it caused a prominent increase in shape-change (Fig.6, lower inset). This effect possibly reflected the action of the cell-membrane permeable H_2O_2 molecule (13) at internal sites while ajoene inhibited its action at peripheral

sites. It is implied that adequate effector action at a minimum of two sites is required to induce platelet aggregation. Concentrations of H_2O_2 used here to induce aggregation of calf GFP are in the range required to induce human platelet aggregation (e.g. Ref.14). A reason for the vast difference between the $S_{0.5}$ of binding to the protein and the $S_{0.5}$ of aggregation may be the presence of powerful catalases in platelets (15, and our unpublished results).

To sum up the results of this paper revealed a parallelism between the modifying effects of ajoene on the ligand-binding properties of the hemoprotein and the modifications of the kinetics of agonist-induced platelet aggregation by the same compound. The irreversible effect of ajoene on the protein conformation and the cooperative character of its interaction provided a rational explanation for similar effects of ajoene on platelet aggregation. Thus the hemoprotein could be an alternative or additional site of ajoene action in platelets. Finally, the results demonstrated the usefulness of the spectrophotometric platelet aggregation assay, together with the sequential shape-change and interaction model, as a powerful tool for kinetic analysis of platelet aggregation and its modulations.

ACKNOWLEDGMENTS. We thank Dr. R. Apitz-Castro, I.V.I.C., Venezuela, and Dr. M.K. Jain, University of Delaware, U.S.A., for NMR spectral data of ajoene, and Jose Jacob for technical help. Molly George helped in the preparation of ajoene. Supported by a grant from the Department of Science & Technology, Government of India, for the scheme "Intercellular Contact Interactions: A Kinetic Approach Employing Blood Platelets". Some of the work reported here will form part of a Ph.D. thesis of L.K.K.

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