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Stimulation of an Esterase Activity of Thrombin by Dequalinium and Its Relationship with Blood Coagulation

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Abstract: Effects on thrombin by an amphipathic cation, dequalinium, which has been recognized as an anticarcinoma agent were investigated with small chromogenic substrates such as Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), H-D-phenylalanyl-L-pipecoyl-L-arginine-p-nitroanilide (S-2238), and Nα-p-tosyl-L-arginine methyl ester (TAME). Among them, only TAME hydrolysis due to an esterase activity of the enzyme was significantly activated to 81% at 20 μM dequalinium in the absence of NaCl. This stimulation became even higher in the presence of 0.2 M NaCl to 3.5-fold at 60 μM dequalinium. This specific activation of thrombin was well correlated with the results of *in vitro* coagulation tests measuring the activated partial thromboplastin time (APTT) and the prothrombin time (PT). It is pertinent, therefore, to suggest that the esterase activity should be examined in addition to the effects on S-2238 hydrolysis when especially any regulators not directed to an active site of thrombin need to be studied. We also expect that dequalinium could be a useful tool for studying structure-function relationship of thrombin and blood coagulation.
Key words: amphipathic cation, blood coagulation, dequalinium, esterase, thrombin.

Thrombin plays pivotal roles in hemostasis by exhibiting two opposite effects in the blood coagulation cascade (Davie *et al.*, 1989; Mann *et al.*, 1990). As a procoagulatory agent, thrombin specifically recognizes and converts fibrinogen into fibrin, which is the basis of the blood clot. The generation of this serine protease from its precursor, prothrombin, is finely regulated by the positive and negative feedback mechanisms in the cascade. The enzyme stimulates its own production by activating factors V and VIII and by converting factors VII and XI from zymogens to active proteases responsible for initiating the extrinsic and the intrinsic pathways, respectively. Thrombin also participates in an anticoagulation mechanism by activating protein C in the presence of thrombomodulin, which eventually removes the activated factors V and VIII by proteolysis (Esmon, 1989). In addition, the enzyme is involved in various biological phenomena such as platelet activation (Oberghen-Schilling and Pouyssegur, 1993), mitosis of fibroblast (Gralnick *et al.*, 1994), and neurite retraction in neurons (Gurwitz and Cunningham, 1988). This multifunctional enzyme, therefore, has become a main target for drug development affecting hemostasis (Stubbs and Bode, 1995). The enzyme exhibits both amidase and esterase activities which have been used to assess the effects of drugs *in vitro* with small chromogenic substrates.

Dequalinium, 1,1′-(1,10-decanediyl)bis-[4-amino-2-methylquinolinium], known as a topical antimicrobial agent, was shown to contain anticarcinoma activity by selective mitochondrial accumulation (Weiss *et al.*, 1987). This amphipathic cation with two positive charges delocalized in the 4-aminoquinaldine rings (Fig. 1) was also a potent inhibitor of protein kinase C (Rotenberg *et al.*, 1990), a calmodulin-stimulated cyclic nucleotide phosphodiesterase (Bodden *et al.*, 1986), and mitochondrial F_1-ATPase (Zhuo *et al.*, 1988). In addition, this compound irreversibly modified the F_1-ATPase in a structure-specific manner upon UV illumination (Zhuo *et al.*, 1993; Paik *et al.*, 1994).

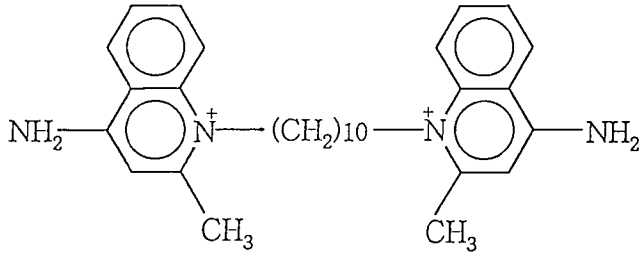

Fig. 1. Structure of Dequalinium.

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We report here the effects of dequalinium on thrombin in the dark with various chromogenic substrates such as Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), H-D-phenylalanyl-L-pipecoyl-L-arginine-p-nitroanilide (S-2238), and Nα-p-tosyl-L-arginine methyl ester (TAME). Also, its effects on blood coagulation are examined with *in vitro* coagulation tests measuring the activated partial thromboplastin time (APTT) and the prothrombin time (PT).

Materials and Methods

Materials

Thrombin from bovine plasma (cat. #T6634), dequalinium, TAME, BApNA, and Tris were purchased from Sigma. S-2238 was obtained from Chromogenix (Sweden). Dade® Thromboplastin IS, Dade® Actin® Activated Cephaloplastin Reagent, and Dade® Ci-Trol® Coagulation Control (level I) were from Baxter Diagnostic Inc.

Esterase activity of thrombin

The esterase activity was assayed with the method of Hummel (1959). A typical reaction was carried out directly in a cuvette which contained 0.5 μM thrombin, 0.8 mM TAME, and various concentrations of dequalinium with 50 mM Tris-Cl (pH 8.0) to a total volume of 0.6 ml in the presence or absence of 0.2 M NaCl. A 10 mM stock of dequalinium was prepared in 50% ethanol. Dequalinium was added to the reaction mixture so as to maintain ethanol concentration in 2.5%. As soon as the substrate was added to the mixture, TAME hydrolysis was continuously monitored as increased absorbance at 247 nm with a Uvikon spectrophotometer 930 (Kontron instruments). The initial rates were obtained from slopes of the curves within 1 min. Since dequalinium interfered with the absorbance at 247 nm in a concentration-dependent manner, the decreases in extinction coefficients of the hydrolyzed products at each concentration of the effector were corrected. The effect of dequalinium was analyzed by comparing the activities of thrombin in the presence of various concentrations of dequalinium as v/v_o, where v and v_o represented the activities in the presence and absence of the effector, respectively.

Amidase activity of thrombin

Both S-2238 and BApNA were used to examine the amidase activity of thrombin. The hydrolyzed products, p-nitroaniline, from both substrates were continuously monitored as increased absorbance at 405 nm. S-2238 was added to a final concentration of 0.5 mM in a 0.6 ml reaction mixture consisting of 50 nM thrombin, dequalinium, and 50 mM Tris-Cl (pH 8.0). When BApNA was used as a substrate, 0.5 μM thrombin and 2 mM substrate were added to the same reaction mixture as S-2238. Other experimental procedures and analysis of data were performed as in the case of esterase activity.

Prothrombin time (PT) test

Prothrombin time was determined with Dade® Thromboplastin IS and Dade® Ci-Trol® Coagulation control (level I) as normal human plasma. The thromboplastin of 0.2 ml was added to 0.1 ml of the control plasma prewarmed at 37℃ for 2 min in the presence and absence of dequalinium. All samples including controls contained 5% ethanol. The durations required for the clot formation were observed manually with the tilt tube method. All tests were performed in duplicate. The results were presented in % activation according to an equation of $[(to-te)/to]\times100$, where to and te represented PT in the absence and presence of dequalinium, respectively.

Activated partial thromboplastin time (APTT) test

The APTT was monitored manually with an Actin® activated cephaloplastin reagent. Actin® (0.1 ml) was preincubated at 37℃ for 1 min then mixed with 0.1 ml of the control plasma and further incubated for 3 min. The time required for fibrin web formation was measured after 0.1 ml of prewarmed 0.02 M $CaCl_2$ was added to the reagent plasma mixture in the presence and absence of dequalinium. The ethanol concentrations remained at 5% in all the tests. The data was analyzed in % activation as in the PT test.

Results and Discussion

Dequalinium effects on amidase and esterase activities of thrombin were observed with S-2238, BApNA, and TAME (Fig. 2). Among them, only the esterase activity measured with TAME as a substrate was significantly stimulated by dequalinium while other amidase activities were either unaffected or slightly inhibited. The esterase activ ity was maximally stimulated to 81% at 20 μM dequalinium and the effect slowly disappeared thereafter. Whereas S-2238 cleaving amidase activity was not affected at all, BApNA cleavage was slightly inhibited to 14% at 75 μM dequalinium. The TAME concentration used in this study was 0.8 mM at which thrombin was already maximally stimulated by a phenomenon of substrate activation. This dequalinium stimulation, hence, was not due to the substrate activation by TAME. It was reported that Nα-toluene-p-sulfonyl arginine-containing esters including TAME were catalyzed

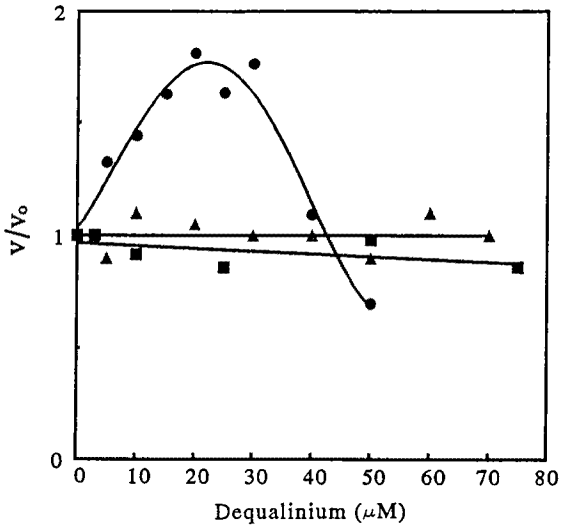

Fig. 2. Dequalinium effects on both amidase and esterase activities of thrombin. The esterase activity was measured with TAME (●) at 247 nm. The amidase activities with S-2238 (▲) and BA-pNA (■) were continuously monitored at 405 nm. The initial rates obtained from slopes of the curves were compared for each substrate as v/v_o, where v and v_o represented the activities in the presence of absence of dequalinium, respectively.

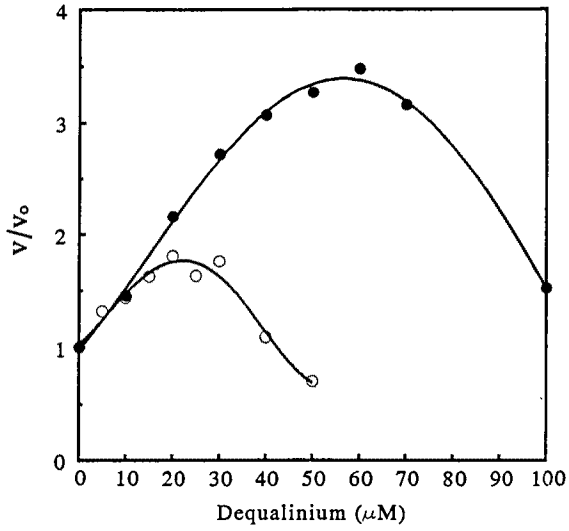

Fig. 3. Influence of Na^+ ion for the dequalinium effect on the esterase activity. The activities of thrombin affected by dequalinium were monitored at 247 nm in the presence (●) and absence (○) of 0.2 M NaCl. The result was shown with v/v_o, where v and v_o indicated the activities in the presence and absence of dequalinium, respectively.

by the enzyme with rate-determining steps influenced by both acylation and deacylation processes while Nα-benzoyl-L-arginine containing esters were hydrolyzed with rates to which deacylation exclusively contributed as a rate-determining step (Curragh and Elmore, 1964). It was clear that the Nα-substituents on the arginine residue affected the accessibility of substrates to an active site of thrombin. This fact indicates that a specific interaction of dequalinium to thrombin accelerates the acylation step in TAME hydrolysis, so that the step can not participate in the rate-determining process any more. A possible dequalinium binding site on thrombin must be distinct from an active site since S-2238 hydrolysis was not affected by the amphipathic cation at all. The dequalinium binding to the enzyme, therefore, can open up the catalytic site for TAME to be accessed. This site can be also different from the Na^+ binding site which has been recognized as regulating S-2238 hydrolysis. It has been reported that thrombin became a "fast" form from its "slow" form upon Na^+ ion binding, which enhanced S-2238 hydrolysis by facilitating both acylation and deacylation steps (Wells and Cera, 1992). It was also suggested that the "fast" form can act as a procoagulant by increased fibrinogen cleavage while the "slow" form can selectively activate protein C, which led thrombin an anticoagulant (Dang *et al.*, 1995).

The dequalinium effect on TAME hydrolysis, therefore, was examined in the presence of 0.2 M NaCl (Fig. 3). It was interesting to note that esterase activity was further stimulated to even 3.5-fold at 60 μM dequalinium in the presence of NaCl. In addition, the concentration required for the maximal stimulation was increased from 20 μM to 60 μM in the presence of the salt. There are two possible explanations for this higher activation compared to the stimulation observed in the absence of NaCl. One is that a specific binding of Na^+ ion which accompanies a conformation change of thrombin (Ayala and Cera, 1994) can lead the enzyme more stably to dequalinium. In addition, this new conformation, which can selectively interfere with the acylation step for TAME, makes dequalinium interact with its site(s) more efficiently. This dequalinium binding can eventually release the interference. In fact, NaCl decreased the esterase activity to 4-fold at a 0.3 M concentration although S-2238 hydrolysis was certainly stimulated with the salt (Berliner and Shen, 1977). This decrease in esterase activity was suggested to be due to the acylation step being affected more effectively. The other possibility would be a simple competition between Na^+ and dequalinium to a common site. The Na^+ ion binding could be protected by dequalinium, which makes the acylation step unaffected. Along with the dequalinium's own effect on the increased acylation rate, this protection could stimulate esterase activity more significantly. Although we tried an irreversible chemical modification of thrombin with dequalinium under UV illumination to obtain information on the effector binding site, the enzyme was not photoinactivated by the reagent. Since dequalinium photoinactivated of F_1-ATPase might require both an α-helical struc-

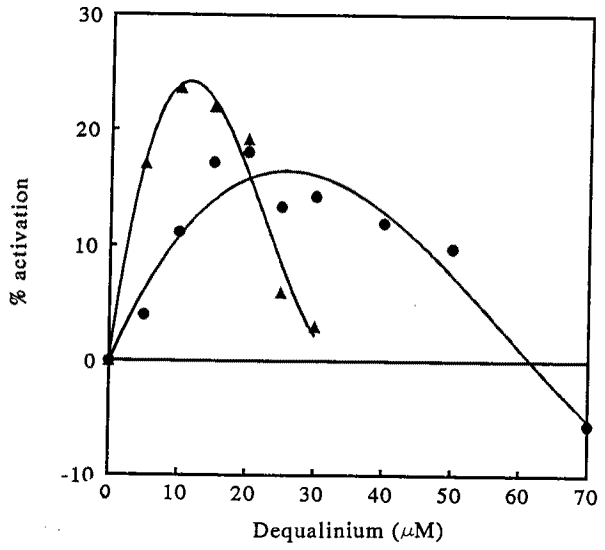

Fig. 4. Dequalinium effects on *in vitro* coagulation tests were examined with APTT (●) and PT (▲). The decreases of durations required for the clot formations were shown as % activation calculated by an equation of [(to−te)/to]×100, where to and te represented APTT and PT in the absence and presence of dequalinium, respectively.

ture and phenylalanine(s) (Zhuo *et al.*, 1993; Paik *et al.*, 1994), it is certainly possible that thrombin can not provide the amphipathic cation to the two conditions to be satisfied simultaneously.

In order to relate the effect of dequalinium on TAME hydrolysis to blood coagulation, APTT and PT tests were performed in the presence of the stimulator (Fig. 4). As one would expect, durations required for fibrin clot formations in both APTT and PT were shortened with somewhat different shapes of the % activation curves. This fact indicates that dequalinium exhibits its effect on the common pathway in the cascade where thrombin takes a part. In the case of the PT test, it responded more quickly to dequalinium, where maximal stimulation of 24% was observed at a 10 μM concentration. The duration for the clot formation in the absence of dequalinium was 45.57 seconds, which was considered as a control. On the other hand, the stimulation of clot formation through the intrinsic pathway assessed with APTT was rather slow in responding with 18% stimulation observed at the 20 μM effector, in which 86.05 seconds were required for the clot formation without the amphipathic cation. The different shapes of the curves might indicate that dequalinium can interact with other factors in the coagulation cascade besides thrombin. Although this possibility can not be completely excluded, the direct correlation between the activation of an esterase activity with TAME and the dose-dependent decrease in both APTT and PT is still very informative. It is pertinent, therefore, to suggest that the esterase activity using TAME as a substrate should be examined in addition to the effects on S-2238 hydrolysis when any regulatory mechanisms for thrombin need to be studied, especially with effectors not directed to the active site. The S-2238 has been one of the most widely used substrates for thrombin research. There have been several examples which definitely affected the fibrin clot formations without influencing S-2238 or related substrate hydrolysis. Thrombin molecules, such as meizothrombin and α-thrombin, which are differently processed from their precursor, and which showed opposite effects on fibrin clot formation as an anticoagulant and a procoagulant, respectively, did not affect S-2238 hydrolysis (Doyle and Mann, 1990). A peptide derived from hirudin (residues 45-64) certainly increased APTT in a dose-dependent manner, which did not inhibit thrombin activity toward tosyl-Gly-Pro-Arg-p-nitroanilide (Maraganore *et al.*, 1989).

Dequalinium, which has bifunctional property due to the two cationic 4-aminoquinaldine rings on both sides of the molecule, might be unique in terms of its effect on esterase activity and fibrin clot formation. There were several compounds studied which stimulated the esterase activity of thrombin. Bile salts such as cholate and glycocholate activated TAME hydrolysis to 1.5-fold and 2.1-fold, respectively, at their 2 mM concentrations (Curragh and Elmore, 1964). Nonionic detergents such as Tween 20, 40, and 80 enhanced the activity to between 1.4-fold and 1.7-fold at 0.3% concentrations (Curragh and Elmore, 1964). Indole stimulated hydrolysis with a K_d of 4.3± 1.8 mM to an "apolar binding site" (Berliner and Shen, 1977). None of these effectors, however, decreased the durations required for fibrin clot formations. In addition, the concentration ranges for the activations were much higher than in the case of dequalinium. This fact indicates that dequalinium specifically binds to thrombin, which might be distinct from the sites of the above compounds.

In conclusion, dequalinium selectively stimulated the TAME-hydrolyzing esterase activity of thrombin, which was well correlated with the decrease in both APTT and PT. We suggest, therefore, that dequalinium can be another useful tool for studying the structure-function relationship of thrombin and blood coagulation.

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