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Activation of the Plasma Kallikrein-Kinin System by *Vibrio vulnificus* Protease

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***Vibrio vulnificus* protease enhanced hypodermic vascular permeability when injected into the dorsal skin of a guinea pig. Enhancement of permeability was observed within 2 min, and the permeability-enhancing reaction terminated at about 10 min postinjection. The permeability-enhancing reaction was greatly augmented by simultaneous injection of a kininase II inhibitor, whereas the reaction was inhibited by soybean trypsin inhibitor, a well-known inhibitor of plasma kallikrein. Furthermore, in vitro activation of plasma prekallikrein to kallikrein by *V. vulnificus* protease was observed. These results indicate that *V. vulnificus* protease enhances vascular permeability through activation of the plasma kallikrein-kinin system which generates bradykinin, factor in edema formation.**

Vibrio vulnificus infections are divided into two groups, a wound-infection group and a primary septicemia group (1, 2). In the former group, edema and erythema form around a new wound in contact with seawater. In the latter group, cutaneous lesions characterized by bullae, erythema, or necrotic ulcer often form on the skin of the limbs. Extreme hemoconcentration due to edema fluid accumulation and death have been observed experimentally in mice injected with living *Vibrio* cells (3, 14). Therefore, the permeability-enhancing or edema formation factor(s) appears to be important in the pathogenesis of the vibrio.

V. vulnificus produces many toxic or pathogenic factors, including cytolysin (5, 9, 15), protease (8, 9, 17), phospholipase A_2 (18), and siderophores (16). Of these factors, cytolysin has been found to possess vascular-permeability-enhancing activity (5, 9, 15). We recently noted that *V. vulnificus* protease stimulates histamine release from mast cells, consequently enhancing hypodermic permeability in rat skin (12, 13). Our unpublished work demonstrated that guinea pig skin, which has less histamine than rat skin, is more sensitive to permeability-enhancing reaction induced by *V. vulnificus* protease. Furthermore, most permeability-enhancing effects of the protease injected into the guinea pig skin, in contrast to the effects of injection into rat skin, were not blocked by the simultaneous injection of the antihistaminic agent (unpublished data). These observations suggest the existence of another permeability-enhancing mechanism which is possibly specific for the guinea pig.

The plasma kallikrein-kinin system, which generates bradykinin, another permeability-enhancing and edema-formation factor, develops well in the guinea pig but poorly in the rat. The first step in the activation of the plasma kallikrein-kinin system is activation of the Hageman factor, which then activates plasma prekallikrein to kallikrein. Plasma kallikrein has two functions: activation of the Hageman factor through a positive feedback process and generation of bradykinin from high-molecular-weight kininogen, followed by permeability enhancement. Bradykinin thus generated is quickly inactivated by kininases I and II. Of the bacterial proteases, a serratial protease is known to activate this system by limited proteolysis of the Hageman factor in a manner similar to its activation of plasma kallikrein because it possesses plasma kallikrein-like substrate specificity (6, 10).

In the present study, *V. vulnificus* protease was found to activate the plasma kallikrein-kinin system both in vitro and in vivo, thus enhancing vascular permeability through bradykinin generation in the guinea pig.

V. vulnificus protease purified as described previously (11) was diluted to various concentrations with 0.9% NaCl, and 0.1 ml of the sample was injected into the dorsal skin of a male Hartley guinea pig (400 to 500 g) which was previously injected with 5% Evans blue (1 ml/kg of body weight) intravenously. For detection of hemorrhage, injection with Evans blue was omitted. At 15 min postinjection, the guinea pig was sacrificed, the dorsal skin was flayed, and the diameter of either the blueing spot, caused by extravasation of Evans blue, or the hemorrhagic spot was measured.

In a similar manner, *V. vulnificus* protease injected intradermally into a guinea pig enhanced vascular permeability and formed a hemorrhagic lesion due to the tissue-destructive effect. These reactions were dose dependent in the range of 0.3 to 10.0 μg (Fig. 1). The permeability-enhancing and hemorrhagic activities of the protease, as well as the histamine-releasing and proteolytic activities previously mentioned (11–13), were lost upon heat treatment (10 min at 60 or 90°C) or upon preincubation with phosphoramidon, a metalloprotease inhibitor (Peptide Institute Inc., Minoh, Japan) (data not shown). When the protease (3.0 or 1.0 μg) was injected into the guinea pig skin a visible blueing spot was formed within 2 min, and the diameter of the blueing spot reached a plateau at 10 min postinjection (Fig. 2). When 1.0 μg of the protease was injected 60, 30 and 15 min before the administration of Evans blue (a total of 3.0 μg of protease was injected), no blueing spot was observed at any injection site (data not shown), indicating the transitory nature of the permeability-enhancing reaction induced by the protease. Hemorrhage was also observed within 2 min after

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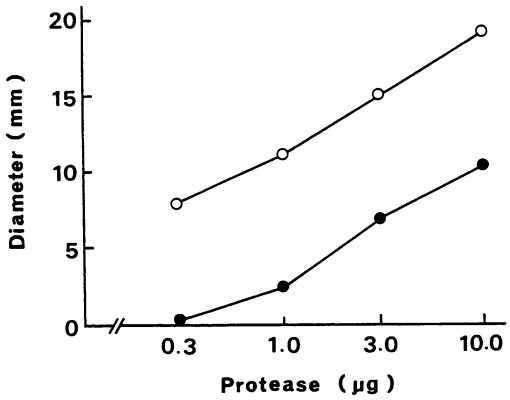

FIG. 1. Permeability-enhancing and hemorrhagic activities of *V. vulnificus* protease in guinea pig skin. *V. vulnificus* protease (0.3 to 10.0 μg) was injected intradermally into the dorsal skin of a guinea pig. At 15 min postinjection, the diameters of the blueing spot (○) and the hemorrhagic spot (●) were measured.

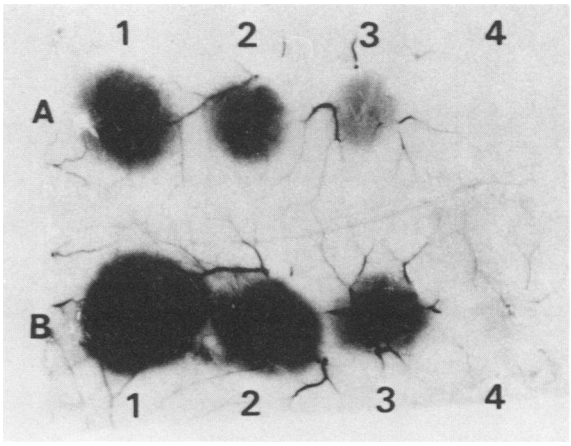

FIG. 3. Effect of a kininase II inhibitor on the premeability-enhancing reaction by *V. vulnificus* protease. (A) protease alone. (B) Protease mixed with 5 nmol of a kininase II inhibitor. *V. vulnificus* protease amounting to 3.0 (1), 1.0 (2), or 0.3 μg (3) or saline alone (4) was injected into the dorsal skin of a guinea pig, and the blueing spot was measured at 15 min postinjection.

protease injection (data not shown). Thus, it is possible that the enhancement of permeability by *V. vulnificus* protease in guinea pig skin is due to release of a mediator, such as histamine or bradykinin, causing the transitory permeability reaction.

Histamine is not a major permeability-enhancing factor in guinea pig skin, in contrast to its significance in rat skin. Thus, the contribution of bradykinin to permeability enhancement in guinea pig skin was investigated. The effect of a kininase II inhibitor, pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (Sigma Chemical Co., Detroit, Mich.), on the permeability-enhancing reaction is shown in Fig. 3. The reaction was greatly augmented when protease was mixed with the inhibitor (5 nmol) as compared with reaction with the protease alone. On the other hand, the enhancement of permeability by the protease was significantly inhibited by the simultaneous injection of 5 nmol of soybean trypsin inhibitor, a well-known inhibitor of plasma kallikrein (type I-S; Sigma) (Fig. 4). Neither inhibitor affected the hemorrhagic and proteolytic activities (data not shown). These results suggest that *V. vulnificus* protease activates the plasma kallikrein-kinin system and that the bradykinin thus generated increases permeability.

To confirm the activation of the plasma kallikrein-kinin system by *V. vulnificus* protease, we investigated the in vitro activation of plasma prekallikrein to kallikrein by enzyme reaction by the method of Uchida et al. (19) with a modification. A 0.2-ml amount of plasma was mixed with 1.8 ml of 20 mM Tris hydrochloride buffer containing 150 mM NaCl and 0.05% Polybrene (pH 8.0). After the addition of 20 μl of 10 mM carbobenzoxy-phenylalanyl-arginyl-4-methyl-coumaryl-7-amide (Z-Phe-Arg-MCA) (Peptide Institute Inc.) and 100 μl of the protease solution the reaction mixture was incubated at 30°C, and the increase in the A_{370} due to release of 7-amino-4-methyl-coumarin (AMC), a degradation product of the substrate, was measured. When guinea pig plasma obtained by the method of Uchida et al. (19) was incubated with *V. vulnificus* protease (10.0 or 5.0 μg), steady and progressive activation of plasma prekallikrein was observed

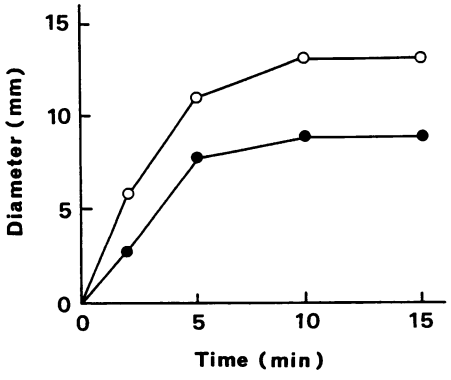

FIG. 2. Time course of permeability-enhancing reaction by *V. vulnificus* protease. *V. vulnificus* protease amounting to 3.0 μg (○) or 1.0 μg (●) was injected periodically into the dorsal skin of a guinea pig.

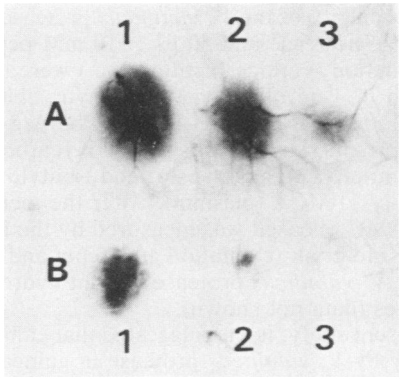

FIG. 4. Effect of soybean trypsin inhibitor, an inhibitor of plasma kallikrein, on the premeability-enhancing reaction by *V. vulnificus* protease. (A) Protease alone. (B) Protease mixed with 5 nmol of soybean trypsin inhibitor. *V. vulnificus* protease amounting to 3.0 (1), 1.0 (2), or 0.3 μg (3) was injected into the dorsal skin of a guinea pig, and the blueing spot was measured at 15 min postinjection.

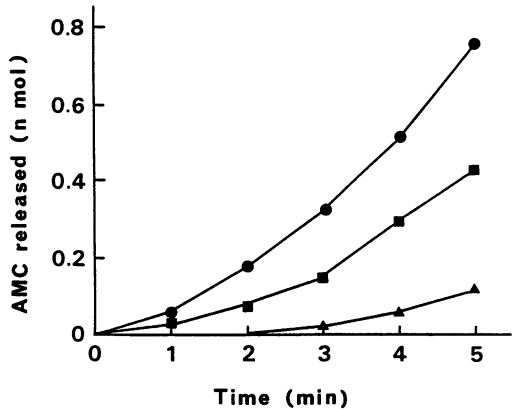

FIG. 5. Activation of plasma prekallikrein by *V. vulnificus* protease in vitro. Guinea pig plasma (0.2 ml) was incubated with *V. vulnificus* protease amounting to 10.0 (●) or 5.0 μg (■) or with saline alone (▲) in the presence of Z-Phe-Arg-MCA, a substrate for plasma kallikrein. The amount of AMC released by degradation of the substrate was then measured.

(Fig. 5). However, very little activation was observed in the absence of the protease. On the other hand, AMC release from the substrate by direct action of *V. vulnificus* protease (10.0 μg) was not detected during the incubation period (data not shown), indicating that *V. vulnificus* protease released AMC indirectly through activation of plasma prekallikrein to kallikrein. In addition, the protease activated human plasma prekallikrein. When normal human plasma (George King Bio-Medical Inc., Overland Park, Kans.) and the protease (5.0 μg) were incubated at 30°C for 5 min, 0.20 nmol of AMC was released. However, in the study using the Hageman factor-deficient or prekallikrein-deficient human plasma (George King Bio-Medical Inc.), AMC release was not observed (data not shown). This in vitro action was also abolished by preincubation with phosphoramidon (data not shown).

Plasma kallikrein, trypsin, and plasmin are known to activate the plasma kallikrein-kinin system by limited proteolysis of the Hageman factor (4). A serratial protease was also reported to activate this system by a similar mechanism because the serratial protease possesses plasma kallikrein-like substrate specificity (6, 10). Thus, we studied the substrate specificity of the *V. vulnificus* protease. A 10.0-μg amount of the protease and 50 μl of 10 mM peptidyl-MCA substrate solution (Peptide Institute Inc.) were incubated at 30°C for 4 h in 4 ml of 100 mM Tris hydrochloride buffer containing 150 mM NaCl (pH 8.0), Z-Phe-Arg-MCA (substrate for plasma kallikrein), t-butyloxycarbonyl-phenyl-alanyl-seryl-arginyl-MCA (trypsin) and t-butyloxycarbonyl-valyl-leucyl-lysyl-MCA (plasmin). After the incubation, the amount of AMC released was measured by the fluorometric method of Kato et al. (excitation at 380 nm and emission at 460 nm) (7). *V. vulnificus* protease did not hydrolyze any of the substrates (data not shown).

In the present study, it was suggested that enhancement of permeability by *V. vulnificus* protease in guinea pig skin is due to bradykinin generation by activation of the plasma kallikrein-kinin system. The activation of plasma prekallikrein by proteases such as plasma kallikrein, trypsin, or plasmin or by a serratial protease is known to occur through the indirect action of the proteases as mentioned above. Although *V. vulnificus* protease activated plasma prekallikrein, the substrate specificity of *V. vulnificus* protease did not resemble that of any of the proteases described above. Therefore, *V. vulnificus* protease may activate plasma prekallikrein by a mechanism different from those of the proteases mentioned above. The precise mechanism of activation is now being investigated at our laboratory, and the results will be published elsewhere.

We have reported that *V. vulnificus* protease enhances vascular permeability in rat skin by stimulating release of histamine from mast cells (12, 13). In addition to stimulating histamine release, the protease appears to activate the plasma kallikrein-kinin system, followed by generation of bradykinin. Since both histamine and bradykinin are permeability-enhancing factors in humans, cutaneous lesions characterized by formation of erythema, edema, or ulcer, which are pathognomonic symptoms of *V. vulnificus* infections, may be attributed to the release of these mediators by protease action. However, *V. vulnificus* cytolysin is also known to increase vascular permeability (5, 9, 15). Thus, additional work is required to determine whether this protease is involved in causing cutaneous lesions.

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