Inhibition of Human Blood Coagulation Factor XIa by $C\bar{1}$ Inhibitor†

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ABSTRACT: The inactivation of activated factor XI (factor XIa) and of its isolated light chain by $C\bar{1}$ inhibitor was studied. Irreversible inhibition was observed in a reaction in which no reversible enzyme–inhibitor complex was formed. The second-order rate constants for the inactivation of factor XIa or its light chain by $C\bar{1}$ inhibitor were 2.3×10^3 and 2.7×10^3 M^{-1} s^{-1}, respectively. High molecular weight kininogen did not affect the rate of inactivation. The nature of the complexes formed between factor XIa or its light chain and $C\bar{1}$ inhibitor was studied by using sodium dodecyl sulfate gradient polyacrylamide slab gel electrophoresis. Under nonreducing conditions, two factor XIa–$C\bar{1}$ inhibitor complexes were observed with apparent molecular weights of 230 000 and 300 000. Reduction of these complexes resulted in the formation of a single band with a molecular weight of 130 000. This band is also formed in the reaction of the isolated light chain of factor XIa with $C\bar{1}$ inhibitor. These results demonstrate that two $C\bar{1}$ inhibitor molecules can become bound to the light chains of a factor XIa molecule. In addition, the mechanism of interaction of factor XIa or its isolated light chain with $C\bar{1}$ inhibitor appears identical, and the rate of inactivation of the enzyme by $C\bar{1}$ inhibitor is very similar. Neither the heavy chain of factor XIa nor high molecular weight kininogen is significantly involved in the inactivation of factor XIa by $C\bar{1}$ inhibitor.

Human blood coagulation factor XI participates in the early or contact phase of blood coagulation (Davie et al., 1979). Factor XI circulates in plasma in an inactive zymogen form. Exposure of human plasma to negatively charged surfaces leads to the activation of factor XII and prekallikrein (Kaplan & Austen, 1970; Cochrane et al., 1973; Revak et al., 1977; Griffin et al., 1977). Activated factor XII then activates factor XI by limited proteolysis (Bouma & Griffin, 1977; Kurachi & Davie, 1977). High molecular weight kininogen serves as a nonenzymatic cofactor in this reaction (Griffin & Cochrane, 1976; Meier et al., 1977). In plasma, high molecular weight kininogen and factor XI form a noncovalent complex, which is required for the binding of factor XI to negatively charged surfaces and for its activation to factor XIa (Thompson et al., 1977). Activated platelets can also promote the proteolytic activation of factor XI (Walsh & Griffin, 1981) and possess specific, high-affinity receptors for factors XI (Greengard et al., 1981, 1986) and XIa (Sinha et al., 1984). Factor XIa then remains surface bound, is protected for inactivation by α_1-antitrypsin (Walsh et al., 1986), and activates factor IX (Wiggins et al., 1977).

Regulation of factor XIa occurs by several protease inhibitors. $C\bar{1}$ inhibitor (Forbes et al., 1970), antithrombin III (Damus et al., 1973), α_1-antitrypsin (Heck & Kaplan, 1974), and α_2-antiplasmin (Saito et al., 1979) have been found to inactivate factor XIa. α_1-Antitrypsin is the major inhibitor for factor XIa, both in a purified system and in plasma (Heck & Kaplan, 1974; Scott et al., 1982). Although our results are in agreement with this, we found $C\bar{1}$ inhibitor to be relatively more important in the inactivation of factor XIa than Scott et al. (1982) (Meijers et al., unpublished results).

$C\bar{1}$ inhibitor is a plasma protease inhibitor which operates as an important regulator of the complement and the contact activation system of plasma. It is an α_2-globulin with a high content of amino sugars (Pensky & Schwick, 1969) and is the only plasma proteinase inhibitor capable of inactivating $C\bar{1}$, the activated first component of complement (Donaldson, 1979; Ziccardi, 1981). $C\bar{1}$ inhibitor inactivates not only $C\bar{1}$ and factor XIa but also plasma kallikrein (Kagan & Becker, 1963; Ratnoff et al., 1969), factor XIIa (Forbes et al., 1970; Schreiber et al., 1973), and plasmin (Ratnoff et al., 1969; Schreiber et al., 1973). In this study, we have examined the inhibition of factor XIa by $C\bar{1}$ inhibitor in a purified system. We have determined the kinetics for this reaction, studied the role of high molecular weight kininogen, and examined the molecular mechanism, both with factor XIa and with its isolated light chain.

Materials and Methods

All chemicals obtained from commercial sources were the best grade available.

Purification of Proteins. High molecular weight kininogen (M_r 110 000),[1] $C\bar{1}$ inhibitor (M_r 110 000), and factor XI (M_r 160 000) were isolated from human plasma as described elsewhere (Kerbiriou & Griffin, 1979; Van der Graaf et al., 1983a; Bouma et al., 1983).

Activation of Factor XI. Factor XIa was prepared from factor XI by using β-factor XIIa (Hageman factor fragment) as described by Van der Graaf et al. (1983a). Separation of factor XIa from β-factor XIIa was performed by affinity chromatography on CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) to which immunopurified anti-factor XII antibodies had been coupled as described elsewhere (Van der Graaf et al., 1982) or by FPLC using a Mono S column (Pharmacia). Before application, the sample was diluted 10-fold in starting buffer (50 mM sodium phosphate, 1 mM EDTA, 0.02% sodium azide, and 0.005% Triton X-100, pH 7.2). This diluted material was applied to a Mono S column at a flow rate of 1–2 mL/min. β-Factor XIIa did not adhere to the column (data not shown). After application, the column

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[1] Abbreviations: M_r, molecular weight; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; PAA, polyacrylamide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; BSA, bovine serum albumin.

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was washed, and subsequently factor XIa was eluted in a 5-mL linear gradient of starting buffer to starting buffer containing 1 M NaCl.

Factor XIa light chain (M_r 30000) was isolated as described elsewhere (Van der Graaf et al., 1983b).

Throughout this paper, molar concentrations of factor XIa are calculated per 80000 molecular weight, for purposes of comparison with the isolated alkylated factor XIa light chain.

Kinetic Studies of the Inactivation of Factor XIa or Its Light Chain by C1 Inhibitor. Factor XIa or its light chain was incubated in the presence or absence of high molecular weight kininogen in 0.05 M Tris, pH 7.4, 0.15 M NaCl, and 0.1% (w/v) bovine serum albumin at 37 °C in plastic tubes. After 5 min, C1 inhibitor was added, and the inactivation of factor XIa amidolytic activity was followed by adding a 25-μL sample from the incubation mixture to a plastic cuvette containing 500 μL of 0.4 mM pyroGlu-Pro-Arg-*p*-nitroanilide (S-2366, KabiVitrum, Stockholm, Sweden) in 0.05 M Tris and 0.15 M NaCl, pH 7.8, at various times. The change in absorbance at 405 nm was followed continuously by using a Cary 210 double-beam spectrophotometer or a Beckman Model 3600 double-beam spectrophotometer. The observed ΔA per minute was converted to the percent of maximum activity by comparison with the ΔA per minute of the sample which did not contain C1 inhibitor.

SDS Gradient PAA Slab Gel Electrophoresis Studies on the Inactivation of Factor XIa or Its Light Chain with C1 Inhibitor. Factor XIa and C1 inhibitor were incubated at 37 °C in a final volume of 130 μL in 0.05 M Tris and 0.15 M NaCl, pH 7.4. After 30 min, a 65-μL sample was added to a SDS solution (final concentration 1% SDS) whereas another 65-μL sample was added to a SDS solution containing β-mercaptoethanol (final concentration 1% SDS and 1.7% β-mercaptoethanol). Both samples were incubated for 5 min at 100 °C and used for analysis by SDS–3–18% gradient PAA slab gel electrophoresis.

Factor XIa light chain and C1 inhibitor were incubated in 0.05 M Tris and 0.15 M NaCl, pH 7.4, in a final volume of 1 mL at 37 °C. At various times, a 5-μL aliquot was withdrawn and tested for factor XIa light chain amidolytic activity. At the same times, a 115-μL sample was added to a SDS solution (final concentration 1% SDS) and analyzed by SDS–3–18% gradient PAA slab gel electrophoresis.

SDS gradient PAA slab gel electrophoresis was performed according to the method of Laemmli (1970) under conditions described by Van der Graaf et al. (1983a). After electrophoresis, the proteins were visualized by Coomassie brilliant blue or silver staining (Weber & Osborn, 1969; Morrisey, 1981). The following standard proteins were included as references on each slab gel: myosin (M_r 200000), β-galactosidase (M_r 116500), phosphorylase (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), and carbonic anhydrase (M_r 30000) (Bio-Rad, Richmond, CA).

Results

Kinetics of Inactivation of Factor XIa or Its Light Chain by C1 Inhibitor. Factor XIa or its light chain was incubated with different concentrations of C1 inhibitor. At various times, samples were withdrawn and analyzed for remaining factor XIa or factor XIa light chain amidolytic activity. Irreversible inhibition was observed both for factor XIa and for its light chain (Figure 1). The values of the apparent pseudo-first-order reaction rate constants, k_{app}, were calculated from this figure and plotted as a double-reciprocal plot of k_{app} and the C1 inhibitor concentration (Figure 2). For the inactivation of factor XIa as well as its light chain by C1 inhibitor, a straight line passing through the origin was obtained (Figure 2), which indicated that the mechanism of the inactivation of factor XIa or its light chain by C1 inhibitor can be regarded as a reaction in which no reversible enzyme–inhibitor complex is formed (Kitz & Wilson, 1962). The apparent second-order rate constants for the reaction of factor XIa or its light chain with C1 inhibitor were determined to be 2.3 × 10^3 and 2.7 × 10^3 M^{-1} s^{-1}, respectively.

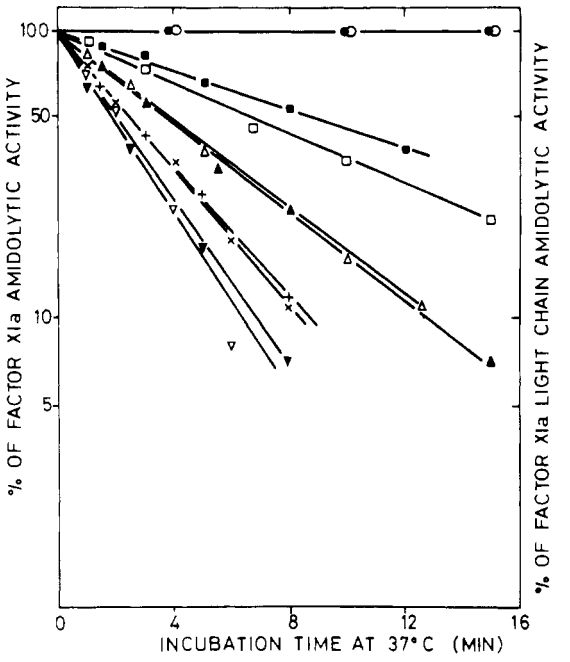

FIGURE 1: Kinetics of inactivation of factor XIa or factor XIa light chain amidolytic activity by C1 inhibitor. Factor XIa or its light chain was incubated at 37 °C with different concentrations of C1 inhibitor in 50 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% BSA. At various times, samples were withdrawn and assayed for remaining factor XIa or its light chain amidolytic activity. The inactivation of factor XIa was assayed at 120 nM in the presence of C1 inhibitor at 0 (●), 0.6 (■), 1.2 (▲), 1.8 (+), and 2.4 μM (▼). The inactivation of the light chain of factor XIa was assayed at 44 nM in the presence of C1 inhibitor at 0 (○), 0.6 (□), 1.2 (△), 1.8 (×), and 2.4 μM (▽).

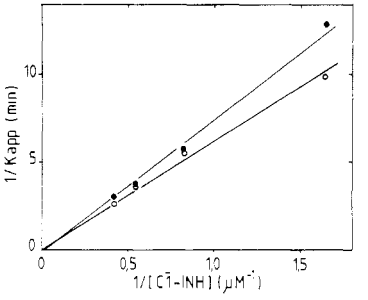

FIGURE 2: Double-reciprocal plot of the pseudo-first-order rate constant of the inactivation of factor XIa (●) or its light chain (○) and the concentration of C1 inhibitor. The pseudo-first-order rate constants were calculated from the data of Figure 1.

Influence of High Molecular Weight Kininogen on the Reaction between Factor XIa and C1 Inhibitor. High molecular weight kininogen forms a bimolecular complex with factor XIa (Thompson et al., 1977). Therefore, it was of interest to investigate whether the rate of factor XIa inactivation by C1 inhibitor is influenced by the high-affinity binding between factor XIa and high molecular weight kininogen. Factor XIa was preincubated with various concentrations of high molecular weight kininogen for 8 min at 37 °C before different amounts of C1 inhibitor were added to the mixture. Then at various times, a sample was withdrawn and added to a cuvette containing 0.4 mM S-2366 to measure factor XIa

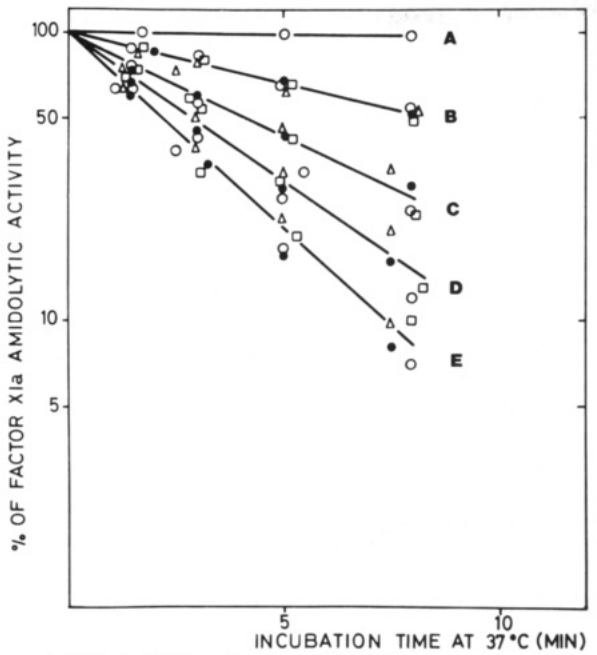

FIGURE 3: Effect of high molecular weight kininogen on the activation rate of factor XIa by C1 inhibitor. Factor XIa at 120 nM was preincubated with different concentrations of high molecular weight kininogen for 8 min in 50 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% BSA. C1 inhibitor was then added at a final concentration of 0 (A), 0.6 (B), 1.2 (C), 1.8 (D), and 2.4 μM (E), and factor XIa amidolytic activity was determined at various times. High molecular weight kininogen was present at 0 (○), 0.6 (●), 1.2 (△), and 1.8 μM (□).

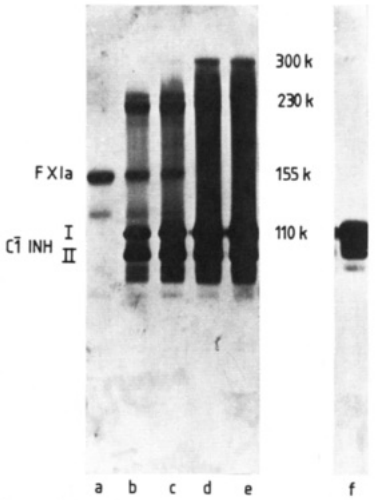

FIGURE 4: SDS gradient PAA slab gel electrophoretic analysis of the reaction between factor XIa and C1 inhibitor. Factor XIa (0.56 μM) was incubated with various concentrations of C1 inhibitor for 30 min at 37 °C. The samples were applied to the gel in the absence of reducing agents. C1 inhibitor was present at 0 (a), 0.14 (b), 0.28 (c), 0.56 (d), and 0.84 μM (e); C1 inhibitor alone at 0.56 μM (f).

amidolytic activity. No effect of high molecular weight kininogen could be observed on the rate of factor XIa inactivation by C1 inhibitor (Figure 3) at any of the concentrations of high molecular weight kininogen or C1 inhibitor examined.

SDS Gradient PAA Slab Gel Electrophoresis Studies of the Reaction between Factor XIa and C1 Inhibitor. The interaction between factor XIa and C1 inhibitor was examined by incubation of factor XIa with various amounts of C1 inhibitor. The mixture was subsequently analyzed by SDS–3–18% gradient PAA slab gel electrophoresis. Under nonreducing conditions, several new bands were observed (Figure 4). At a molar ratio of factor XIa to C1 inhibitor below 1, two new bands were observed with molecular weights of approximately 230 000. At a molar ratio of factor XIa to C1 inhibitor above 1, an additional band at M_r 300 000 is also visible. A faint staining is observed between the M_r 300 000 band and the C1 inhibitor band, which may be due to dissociation of the complex during electrophoresis. In addition, the factor XIa band has disappeared, and in concert with this, a new band has been formed in the C1 inhibitor band II position. These findings suggested that factor XIa had produced a lower molecular weight derivative of the C1 inhibitor band I, indicated here as band II. This band was also observed for the reaction of kallikrein with C1 inhibitor (Van der Graaf et al., 1983a).

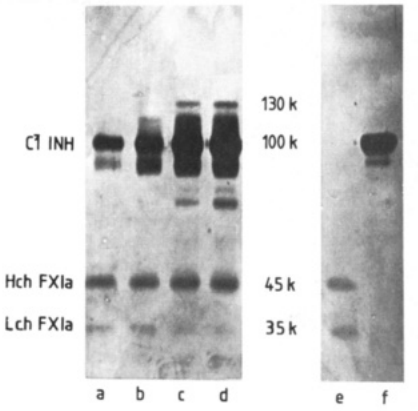

FIGURE 5: SDS gradient PAA slab gel electrophoretic analysis of the reaction between factor XIa and C1 inhibitor. Factor XIa (0.56 μM) was incubated with various concentrations of C1 inhibitor for 30 min at 37 °C. The samples were reduced prior to electrophoresis. C1 inhibitor was present at 0.14 (a), 0.28 (b), 0.56 (c), 0.84 (d), and 0 μM (e); C1 inhibitor alone at 0.56 μM (f).

Analysis of the same samples under reducing conditions indicated the formation of a band with an approximate molecular weight of 130 000. In concert with the formation of the M_r 130 000 band, a decrease in the intensity of the light chain of factor XIa was apparent, whereas the density of the heavy chain remained unaltered during the incubation (Figure 5). These results suggest that the band with an apparent molecular weight of 130 000 represented a complex between C1 inhibitor and the light chain of factor XIa. It also indicates that no SDS-stable interactions are formed between C1 inhibitor and the heavy chain of factor XIa.

Studies on the Interaction of the Light Chain of Factor XIa with C1 Inhibitor. C1 inhibitor was incubated with the light chain of factor XIa. At different times, samples were taken and subjected to SDS gradient PAA slab gel electrophoresis. In time, the band of the light chain of factor XIa decreases, while a new band with a molecular weight of 135 000 is formed (Figure 6). The formation of this band indicated that C1 inhibitor formed a 1:1 molar complex with the light chain of factor XIa. The C1 inhibitor band II increased in time, and in analogy with the interaction of factor XIa and C1 inhibitor, the light chain of factor XIa also produced an inactive C1 inhibitor form.

Discussion

The inhibition of factor XIa and its light chain by C1 inhibitor was studied. Kinetic data indicate that the apparent pseudo-first-order rate constant for the reaction between factor XIa or its light chain increases linearly with the C1 inhibitor concentration. Hence, it can be inferred that the inactivation reaction does not involve the formation of a reversible enzyme–inhibitor complex (Kitz & Wilson, 1962). The second-order rate constant for the inactivation of factor XIa by C1 inhibitor is 2.3×10^3 M^{-1} s^{-1}. The second-order rate

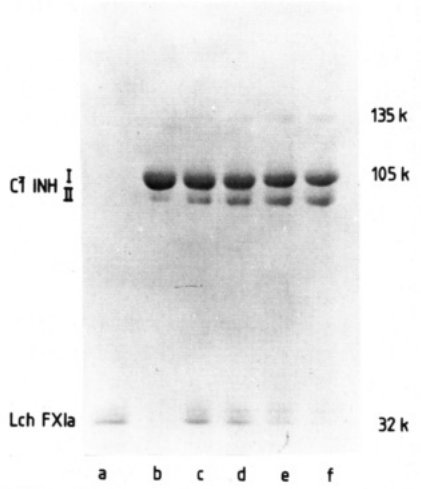

FIGURE 6: SDS gradient PAA slab gel electrophoretic analysis of the reaction between factor XIa light chain and C1 inhibitor. Factor XIa light chain (0.63 μM) was incubated with C1 inhibitor (0.78 μM) at 37 °C. At various times, aliquots were taken and assayed for amidolytic activity or applied to the gel in the absence of reducing agents. In the first two lanes, factor XIa light chain alone (a, 100% amidolytic activity) and C1 inhibitor alone (b) are shown. Time points are 4 min (c, 60%), 10 min (d, 33%), 25 min (e, 13%), and 120 min (f, 0%).

constant for the inactivation of the light chain is 2.7×10^3 M^{-1} s^{-1}, which is only slightly different from factor XIa. This suggests that the heavy chain of factor XIa does not significantly affect the rate of factor XIa inactivation by C1 inhibitor.

Since factor XIa possesses a high-affinity binding site for high molecular weight kininogen, it was of interest to study the effect of high molecular weight kininogen on the rate of inactivation of factor XIa by C1 inhibitor. In contrast to previous reports (Scott et al., 1982), we were unable to demonstrate any detectable effect of high molecular weight kininogen on the rate of inactivation of factor XIa by C1 inhibitor (Figure 3). This agrees with the observation that the heavy chain of factor XIa, which provides the high-affinity binding site for high molecular weight kininogen (Van der Graaf et al., 1983b), does not significantly affect the rate of factor XIa inactivation by C1 inhibitor. High molecular weight kininogen is also known to be a substrate for factor XIa (Scott et al., 1985; van Iwaarden et al., unpublished results). Competition between high molecular weight kininogen and C1 inhibitor for the active site of factor XIa could influence the rate of factor XIa inactivation by C1 inhibitor. Therefore, in our experiments, high molecular weight kininogen was preincubated with factor XIa before addition of C1 inhibitor. Under these conditions, a complex between factor XIa and high molecular weight kininogen is formed (Bouma et al., 1983). Nonetheless, no influence of high molecular weight kininogen on the inactivation rate was observed.

The molecular mechanism of the reaction of factor XIa and C1 inhibitor was studied with SDS gradient polyacrylamide slab gel electrophoresis (Figure 4). Purified C1 inhibitor demonstrated two bands on analysis by SDS gradient polyacrylamide slab gel electrophoresis; a major band designated band I with a molecular weight of 110 000 and a minor band designated band II with a molecular weight of 94 000. The concentration of band II relative to band I was approximately 10% or less. Similar SDS band patterns of C1 inhibitor on nonreduced gels were observed by Harpel and Cooper (1975). Upon incubation with factor XIa, two higher molecular weight bands appear, 230 000 and 300 000. These are the approximate sums of the molecular weights of one C1 inhibitor and one factor XIa molecule and of two C1 inhibitors and one factor XIa molecules, respectively. Previous studies have shown that both light chains of factor XIa incorporate [^{3}H]diisopropyl phosphorofluoridate and react with antithrombin III (Kurachi & Davie, 1977), indicating the presence of an active site on each light chain. Our results therefore suggest that both active sites on the factor XIa molecule become bound to C1 inhibitor. The complexes appear to be stable in SDS, indicative for a covalent bond between protease and inhibitor, but there was some staining between the complex bands and the native protein bands. This is most likely caused by dissociation of part of the complex during electrophoresis.

The relative abundance of the C1 inhibitor band II is increased in the presence of factor XIa. This has been shown before for the reaction of kallikrein and C1 inhibitor (Van der Graaf et al., 1983a). This band was generated from band I and was unable to form a complex with kallikrein. The results obtained after reduction of the factor XIa–C1 inhibitor mixtures indicated that the complex is formed through covalent interaction between the light chain of factor XIa and C1 inhibitor. Conclusive evidence for this was provided by SDS–PAA gel electrophoretic analysis of mixtures of the isolated light chain of factor XIa and C1 inhibitor. A complex of C1 inhibitor–factor XIa light chain is seen on gels with a molecular weight of 135 000 which is almost the same molecular weight as the complex observed on the reduced gel of factor XIa–C1 inhibitor mixtures.

The results of this study demonstrated that no SDS-stable interactions exist between the heavy chain of factor XIa and C1 inhibitor. The mechanism of interaction between factor XIa or its light chain and C1 inhibitor appeared to be identical. Also, the rate constants for the inactivation both of factor XIa and of its light chain by C1 inhibitor are very similar. Hence, the heavy chain region of factor XIa is not significantly involved in the inactivation of factor XIa by C1 inhibitor, and no influence of high molecular weight kininogen on this reaction was observed. Recently, we studied the interaction of factor XIa or its light chain by α_1-antitrypsin (Meijers et al., unpublished results). As with C1 inhibitor, no major role was observed for the heavy chain region of factor XIa in this reaction.

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Registry No. C1, 80295-38-1; factor XIa, 37203-61-5.

REFERENCES

Bouma, B. N., & Griffin, J. H. (1977) *J. Biol. Chem. 252*, 6432–6437.

Bouma, B. N., Vlooswijk, R. A. A., & Griffin, J. H. (1983) *Blood 62*, 1123–1131.

Cochrane, C. G., Revak, S. D., & Wuepper, K. D. (1973) *J. Exp. Med. 138*, 1564–1583.

Damus, P. S., Hicks, M., & Rosenberg, R. D. (1973) *Nature (London) 246*, 355–357.

Davie, E. W., Fujikawa, K., Kurachi, K., & Kisiel, W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 227–318.

Donaldson, V. H. (1979) in *The Chemistry and Physiology of the Human Plasma Proteins* (Bing, D. H., Ed.) pp 369–383, Pergamon Press, New York.

Forbes, C. D., Pensky, J., & Ratnoff, O. D. (1970) *J. Lab. Clin. Med. 76*, 809–815.

Greengard, J. S., Walsh, P. N., & Griffin, J. H. (1981) *Blood 58*, 688 (Abstr).

Greengard, J. S., Heeb, M. J., Ersdal, E., Walsh, P. N., & Griffin, J. H. (1986) *Biochemistry 25*, 3884–3890.

Griffin, J. H., & Cochrane, C. G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2554–2558.
Griffin, J. H., & Cochrane, C. G. (1979) *Semin. Thromb. Hemostasis 5*, 254–273.
Harpel, P. C., & Cooper, N. R. (1975) *J. Clin. Invest. 55*, 593–604.
Heck, L. W., & Kaplan, A. P. (1974) *J. Exp. Med. 140*, 1615–1630.
Kagan, L. J., & Becker, E. L. (1963) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 20*, 613.
Kaplan, A. P., & Austen, K. F. (1970) *J. Immunol. 105*, 802–811.
Kerbiriou, D. M., & Griffin, J. H. (1979) *J. Biol. Chem. 254*, 12020–12027.
Kitz, R., & Wilson, J. B. (1962) *J. Biol. Chem. 237*, 3245–3249.
Kurachi, K., & Davie, E. W. (1977) *Biochemistry 16*, 5831–5839.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Meier, H. L., Pierce, J. V., Colman, R. W., & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 18–31.
Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Nagasawa, S., Takahashi, H., Koida, M., Suzuki, T., & Schoenmakers, J. G. (1968) *Biochem. Biophys. Res. Commun. 32*, 644–649.
Pensky, J., & Schwick, H. G. (1969) *Science (Washington, D.C.) 163*, 698–699.
Ratnoff, O. D., Pensky, J., Ogston, D., & Naff, G. B. (1969) *J. Exp. Med. 129*, 315–331.
Revak, S. D., Cochrane, C. G., & Griffin, J. H. (1977) *J. Clin. Invest. 59*, 1167–1175.
Saito, H., Goldsmith, G. H., Moroi, M., & Aoki, N. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2013–2017.
Schreiber, A. D., Kaplan, A. P., & Austen, K. F. (1973) *J. Clin. Invest. 52*, 1402–1409.
Scott, C. F., Schapira, M., James, H. L., Cohen, A. B., & Colman, R. W. (1982) *J. Clin. Invest. 69*, 844–852.
Scott, C. F., Silver, L. D., Turdon, A. D., & Colman, R. W. (1985) *J. Biol. Chem. 260*, 10856–10863.
Sinha, D., Seaman, F. S., Koshy, A., Knight, L. C., & Walsh, P. N. (1984) *J. Clin. Invest. 73*, 1550–1556.
Thompson, R. E., Mandle, R. J., Jr., & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 1376–1380.
Van der Graaf, F., Keus, J. F. A., Vlooswijk, R. A. A., & Bouma, B. N. (1982) *Blood 59*, 1225–1233.
Van der Graaf, F., Koedam, J. A., Griffin, J. H., & Bouma, B. N. (1983a) *Biochemistry 22*, 4860–4866.
Van der Graaf, F., Greengard, J. S., Bouma, B. N., Kerbiriou, D. M., & Griffin, J. H. (1983b) *J. Biol. Chem. 258*, 9669–9675.
Walsh, P. N., & Griffin, J. H. (1981) *Blood 57*, 106–118.
Walsh, P. N., Sinha, D., Koshy, A., Seaman, F. S., & Bradford, H. (1986) *Blood 68*, 225–230.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4414.
Wiggins, R. C., Bouma, B. N., Cochrane, C. G., & Griffin, J. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4636–4640.
Ziccardi, R. J. (1981) *J. Immunol. 126*, 1769–1773.

Inhibition of β-Bungarotoxin Binding to Brain Membranes by Mast Cell Degranulating Peptide, Toxin I, and Ethylene Glycol Bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic Acid†

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ABSTRACT: The presynaptically active snake venom neurotoxin β-bungarotoxin (β-Butx) is known to affect neurotransmitter release by binding to a subtype of voltage-activated K^+ channels. Here we show that mast cell degranulating (MCD) peptide from bee venom inhibits the binding of ^{125}I-labeled β-Butx to chick and rat brain membranes with apparent K_i values of 180 nM and 1100 nM, respectively. The mechanism of inhibition by MCD peptide is noncompetitive, as is inhibition of ^{125}I-β-Butx binding by the protease inhibitor homologue from mamba venom, toxin I. β-Butx and its binding antagonists thus bind to different sites of the same membrane protein. Removal of Ca^{2+} by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid inhibits the binding of ^{125}I-β-Butx by lowering its affinity to brain membranes.

β-Bungarotoxin (β-Butx)[1] is a snake venom neurotoxin with a Ca^{2+}-dependent phospholipase A_2 activity (Wernicke et al., 1975; Strong et al., 1976; Howard & Gundersen, 1980). The toxin has a M_r of 20 500 and consists of two subunits (Kelly & Brown, 1974). The A subunit (M_r 13 500) carries the phospholipase A_2 activity; the B subunit (M_r 7000) has sequence homology to protease inhibitors (Kondo et al., 1978a,b; Lee, 1979). β-Butx inhibits neurotransmission by interacting with the presynaptic terminal (Abe et al., 1976, 1977). In chick brain, the toxin binds to a membrane protein of M_r 430 000, which contains smaller polypeptide subunits (Rehm & Betz, 1982–1984). Electrophysiological and binding data

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[1] Abbreviations: β-Butx, β-bungarotoxin; MCD peptide, mast cell degranulating peptide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography.

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