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EFFECT OF NEUROTROPIN® ON THE ACTIVATION OF THE PLASMA KALLIKREIN-KININ SYSTEM

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Abstract—Bradykinin (BK), an important mediator of allergic reactions and pain induction, is released by the activation of the plasma kallikrein-kinin (K-K) cascade. Neurotropin® is a biological material obtained from inflamed rabbit skin inoculated with vaccinia virus and is widely used clinically in Japan as an effective agent for these disorders. Since its mechanism of action is not clearly known, we have investigated the effects of Neurotropin on the human plasma K-K system. In dextran sulfate-activated plasma, Neurotropin inhibited the formation of BK, the cleavage of high molecular weight kininogen (HK) and the formation of kallikrein–C1 inhibitor and activated coagulation factor XII (FXIIa)–C1 inhibitor complexes. Experiments using purified enzyme of the K-K cascade indicated that Neurotropin inhibited surface-mediated activation of coagulation factor XII (FXII) and the activation of prekallikrein by FXIIa. Neurotropin also inhibited the binding of FXII and HK to the activating surface. These data suggest that the ameliorating effects of Neurotropin in allergic disorders and pain syndromes may be related to this ability to inhibit activation of the K-K cascade and consequently the formation of BK.

Neurotropin® is a biologic product extracted from inflamed rabbit skin inoculated with vaccinia virus, which is marketed in Japan as an agent effective in both allergic diseases and chronic pain. The extract is deproteinated and includes a variety of low molecular weight natural substances (i.e. amino acids, peptides, nucleic acids, glyco-compounds, etc.) The active moiety of Neurotropin, however, has not been identified. Neurotropin has been shown to inhibit the wheal and flare reactions in response to allergen in atopic individuals [1] and provides pain relief [2, 3] thought to be due to an inhibitory effect upon the kinin-forming pathways [4–6]. In the current study, we have assessed the effects of Neurotropin upon each step of the human plasma kinin-forming system (contact activation) and have demonstrated that it is the first pharmacologic agent that inhibits bradykinin (BK§) formation and the cleavage of high molecular weight kininogen (HK) by agents that initiate the cascade. We also demonstrated that Neurotropin is not an enzyme antagonist, but acts to inhibit the surface-dependent coagulation factor XII (FXII) autoactivation and the binding of FXII and HK to initiating surfaces. As a result, it inhibits the conversion of prekallikrein to kallikrein.

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§ Abbreviations: BK, bradykinin; K-K, kallikrein-kinin; HK, high molecular weight kininogen; FXI, coagulation factor XI; FXII, coagulation factor XII; FXIIa, activated coagulation factor XII; IgG, immunoglobulin γ; SBTI, soy bean trypsin inhibitor; LBTI, lima bean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; HC, heavy chain; and LC, light chain.

MATERIALS AND METHODS

Materials

Rabbit antisera to BK (Dr. L. Arbeit, Department of Medicine, SUNY at Stony Brook, NY), to human C1 inhibitor (Dr. B. Ghebrehiwet, Department of Medicine, SUNY at Stony Brook, NY), to human prekallikrein and to FXII (Dr. P. Harpel, Division of Hematology-Oncology, Department of Medicine and Specialized Center of Research in Thrombosis, New York Hospital-Cornell Medical Center, NY), and mouse monoclonal antibody to human prekallikrein (Dr. R. W. Colman, Department of Medicine, Temple University of Medicine, Philadelphia, PA) were obtained as indicated. Monoclonal antibody to the light chain (LC) of human HK was purified from mouse hybridoma cell culture supernatants [7, 8]. Purified human FXII, activated coagulation factor XII (FXIIa) and HK were obtained from Enzyme Research Laboratories, South Bend, IN. Human prekallikrein and kallikrein were purified from fresh normal plasma as described below. Affi-gel 10, Econo-Pac 10DG and Q-Sepharose, SP-Sephadex C-50 and Sephadex G-200 were obtained from Bio-Rad Laboratories, Richmond, CA, and Pharmacia Fine Chemicals, Uppsala, Sweden, respectively. Protein A column kits and Iodo-beads were from Pierce Chemicals,

Rockford, IL. Dextran sulfate (average mol. wt 500,000) and goat anti-mouse immunoglobulin γ(IgG) antibody conjugated with alkaline phosphatase were purchased from Serva, Heidelberg, Germany, and Jackson Immunoresearch Laboratories, West Grove, PA, respectively. Soy bean trypsin inhibitor (SBTI), lima bean trypsin inhibitor (LBTI), *p*-nitrophenyl phosphate and bradykinin were products of the Sigma Chemical Co., St. Louis, MO. ^{125}I-Tyr^{8}-bradykinin and Na^{125}I were obtained from New England Nuclear, Boston, MA. Alkaline phosphatase and Chromozym PK were purchased from Boehringer Mannheim GmbH, Germany. BCIP/NBT Phosphatase Substrate System (substrate kit for alkaline phosphatase) and S-2302 were obtained from Kirkegaard & Perry Laboratories, Gaithersburg, MD, and KabiVitrum, Franklin, OH, respectively. Immulon 1 microplates were obtained from Dynatech Laboratories, Chantilly, VA. Human pooled plasma was purchased from George King, Overland Park, KS. Neurotropin used as a test substance was obtained from the Nippon Zoki Pharmaceutical Co. Ltd., Japan.

Purification of human plasma prekallikrein and kallikrein

All procedures for the purification of human plasma prekallikrein were performed at room temperature in order to prevent cold activation of the kallikrein-kinin (K-K) cascade. Fresh human plasma (100 mL) containing 0.4% sodium citrate, 15 mM benzamidine hydrochloride, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.036% polybrene was used as a starting material and the first three purification steps, anion (Q-Sepharose) and cation (SP-Sephadex) exchange column chromatography and gel filtration (Sephadex G-200), were performed according to the method of Silverberg and Kaplan [9]. After gel infiltration, the preparation was next applied to an affinity column of Affi-gel 10 (1.5 × 3 cm) conjugated with anti-human prekallikrein monoclonal antibody and equilibrated with 50 mM sodium phosphate buffer, pH 7.4. After extensive washing of the column by 50 mM sodium phosphate buffer, pH 7.4, containing 0.5 M sodium chloride, bound protein was eluted by 3 M potassium thiocyanate containing 0.1 mM EDTA, 0.1 mM PMSF, 0.02% sodium azide, 15 mM benzamidine hydrochloride and 0.01% Triton X-100. Eluted prekallikrein was dialyzed against 50 mM sodium acetate, pH 8.3, and then passed through a Protein A column to remove the contaminating IgG. IgG-free prekallikrein was then dialyzed against 10 mM sodium acetate buffer, pH 5.0. The coagulant activity of purified prekallikrein was determined as described by Proctor and Rapaport [10] and the protein concentration was determined according to the method of Bradford [11]. Purified kallikrein was obtained by the activation of prekallikrein by FXIIa as described previously [9]. The purified kallikrein obtained was then dialyzed against 10 mM sodium acetate buffer, pH 5.0. Both of the purified proteins were stored at −70° until used.

Purification of the light and heavy chain of HK

The light chain and heavy chain of HK were obtained from the digestion of purified HK (6 mg) cleaved by 60 μg of purified kallikrein according to the method described by Kerbiriou and Griffin [12].

Activation of the plasma K-K system by dextran sulfate

Pooled human normal plasma was thawed at 37° immediately before use. The activating reaction was started by the addition of dextran sulfate to the reaction mixture at 37° in the presence of Neurotropin and 1,10-phenanthroline as described below. Pooled plasma was used only one time, and was neither refrozen nor reused. To determine the concentration of 1,10-phenanthroline for the stabilization of kinin formed in plasma, BK was incubated with various concentrations of 1,10-phenanthroline in plasma containing 0.5 mg/mL SBTI, 0.167 mg/mL aprotinin and 0.4 mg/mL polybrene at 37°. Since we found that plasma BK was stable at 5 mM 1,10-phenanthroline, we routinely employed this concentration.

Quantitation of plasma BK

Dextran sulfate-activated plasma was processed according to Minami *et al.* [13] in order to quantitate BK. The concentration of kinin was determined by radioimmunoassay in which ^{125}I-Tyr^{8}-BK was used as a tracer.

Immunoblotting of HK

Immunoblotting of HK was performed according to Reddigari and Kaplan [14] utilizing mouse anti-LC of HK monoclonal antibody.

Preparation of anti-human C1 inhibitor antibody conjugated with alkaline phosphatase

Alkaline phosphatase in 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride was made 0.2% with respect to glutaraldehyde, incubated for 1 hr at room temperature, and the same weight of rabbit anti-human C1 inhibitor in the same buffer was added. The mixture was incubated for 1 hr at room temperature. The conjugation was terminated by the addition of glycine (67 mM final concentration) to the reaction mixture. The sample was dialyzed against 50 mM Tris–HCl buffer, pH 7.4, containing 0.15 M sodium chloride, 1 mM magnesium chloride and 0.02% sodium azide for 16 hr. Bovine serum albumin (BSA) was added to a final concentration of 10 μg/mL, and the sample was stored at −70°.

Detection of plasma kallikrein–C1 inhibitor and FXIIa-C1 inhibitor complexes

The detection of plasma kallikrein–C1 inhibitor and FXIIa–C1 inhibitor complexes was performed using anti-human prekallikrein, anti-human FXII antisera, and anti-human C1 inhibitor antisera conjugated with alkaline phosphatase employed in the double antibody sandwich enzyme-linked immunosorbent assay (ELISA) methods described by Lewin *et al.* [15] and Kaplan *et al.* [16].

Activation of purified prekallikrein

(a) By FXIIa without dextran sulfate. A modification of the method described by Hojima *et al.*

[17] was used. Prekallikrein (1.25 μM) was incubated with 1.25 nM FXIIa in 50 mM Tris–HCl buffer, pH 8.0, containing 0.005% BSA, 0.1 M sodium chloride and 0.01% Triton X-100 for 5 min at 37°. After incubation, kallikrein activity in the reaction mixture was measured by incubation with synthetic substrate Chromozym PK at 37° as described [9].

(b) By FXIIa with dextran sulfate. Prekallikrein (1.25 μM) was incubated with 0.25 nM FXIIa in the same buffer described above containing 5 μg/mL dextran sulfate. Activated kallikrein was assayed as described.

Activation of purified FXII

(a) Autoactivation of FXII. FXII (1.25 μM) was incubated with 2.5 nM FXIIa in 50 mM Tris–HCl buffer, pH 8.0, containing 0.005% BSA, 0.1 M sodium chloride, 0.01% Triton X-100 and 5 μg/mL dextran sulfate at 37°. After several time points of the incubation, FXIIa generated was assayed by incubation with 0.5 mM S-2302 in 50 mM Tris–HCl buffer, pH 8.0, containing 0.05% BSA, 0.15 M sodium chloride and 0.01% Triton X-100 at 37°. Apparent first and second order rate constants were obtained by using the equations described by Cameron *et al.* [18] and Tankersley and Finlayson [19].

(b) By kallikrein without dextran sulfate. FXII (2.5 μM) was incubated with 0.25 μM kallikrein in 50 mM Tris–HCl buffer, pH 8.0, containing 0.005% BSA, 0.1 M sodium chloride, and 0.01% Triton X-100 at 37°. After the incubation, the sample was assayed for generated FXIIa by incubating with 1 mM S-2302 in 50 mM Tris–HCl buffer, pH 8.0, containing 0.005% BSA, 0.15 M sodium chloride, 100 μg/mL aprotinin and 0.01% Triton X-100 at 37°.

(c) By kallikrein with dextran sulfate. FXII (1.25 μM) was incubated with 5 nM kallikrein in the same buffer described above plus 50 μg/mL dextran sulfate at 37°. The activity of FXIIa formed was assayed by same method described above.

Assay of kallikrein activity

Kallikrein activity and the inhibitory effect of tested material were determined spectrophotometrically using synthetic substrates as described above. Alternatively kallikrein (5 nM) was incubated with 0.5 μM HK in 50 mM Tris–HCl buffer, pH 8.0, containing 0.005% BSA, 0.1 M sodium chloride and 0.01% Triton X-100 at 37°. At various time points, 5 μL of the reaction mixture was transferred into 45 μL of sodium dodecyl sulfate (SDS) buffer (25 mM Tris–HCl buffer, pH 6.8, containing 17 mM SDS, 0.68 M glycerol and 0.01% bromophenol blue), denatured, and used for immunoblotting as described above.

Assay of FXIIa activity

FXIIa was incubated with 1 mM S-2302 in 50 mM Tris–HCl buffer, pH 8.0, containing 0.005% BSA, 0.15 M sodium chloride and 0.01% Triton X-100 at 37°.

Binding of FXII, FXIIa and HK to kaolin

Approximately 100,000 cpm of radiolabeled FXII, FXIIa or HK (FXII 7,800,000 cpm/μg, FXIIa 900,000 cpm/μg and HK 4,100,000 cpm/μg) were incubated with 1 mg of kaolin in 25 nM Tris–HCl buffer, pH 8.0, containing 0.14% BSA, 0.115 M sodium chloride and 0.01% Triton X-100 in the presence or absence of 0.65 μg/mL unradiolabeled FXII and FXIIa or 1.6 μg/mL unradiolabeled HK. After a 10-min incubation, the mixtures were centrifuged and the bound radioactivity was quantitated using a γ-counter (Type 1282 Compugamma CS counter, LKB).

Binding of prekallikrein to HK

Five hundred microliters of 25 μg/mL HK was incubated with 1 mg of kaolin for 30 min. After centrifugation, kaolin was incubated with 500 μL of 1% non-fat milk for 30 min and centrifuged again. Kaolin was then treated with 500 μL of labeled prekallikrein (400,000 cpm/mL, 530,000 cpm/μg) in 1% non-fat milk. After a 10-min incubation, the mixture was centrifuged and the radioactivity in the pellet was determined.

^{125}I-Labeling of proteins

^{125}I-Radiolabeling reactions of proteins were performed by the use of Iodo-beads. After the coupling reaction, the radiolabeled protein and free ^{125}I were separated using an Econo-Pac 10DG desalting column.

RESULTS

Activation of the kinin-forming pathway in plasma by dextran sulfate

The formation of BK in plasma incubated with dextran sulfate was determined in the presence or absence of 5 mM 1,10-phenanthroline. The accumulation of BK was maximum (about 600 ng/mL) at 1–2 min in the presence of 20 μg/mL or 100 μg/mL dextran sulfate plus 1,10-phenanthroline. BK generation induced by 100 μg/mL dextran sulfate peaked at 2–3 min (about 300 ng/mL) in the absence of 1,10-phenanthroline, and the kinin was degraded over the ensuing 10 min. The BK level did not increase in the presence of 20 μg/mL dextran sulfate without 1,10-phenanthroline (data not shown).

Effect of Neurotropin on BK formation in dextran sulfate-activated plasma

The effect of Neurotropin on BK formation was determined at the peak time points (1 or 2 min) for each condition tested above. Neurotropin progressively inhibited the formation of BK at 20 μg/mL dextran sulfate plus 1,10-phenanthroline and reached 60% at a Neurotropin concentration of 100 μg/mL (Fig. 1). Inhibition (39%) was also observed using 100 μg/mL dextran sulfate without 1,10-phenanthroline at 100 μg/mL Neurotropin. No significant inhibition was observed with 100 μg/mL dextran sulfate used in the presence of 1,10-phenanthroline.

Since we observed an inhibitory effect on BK formation, we also determined whether inhibition of HK cleavage paralleled this result for each of the conditions tested. Immunoblotting of HK was performed as described in Materials and Methods using the same incubation and concentration

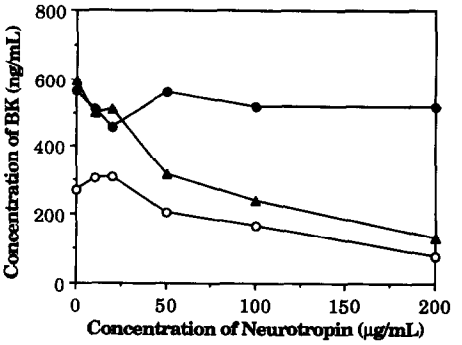

Fig. 1. Effect of Neurotropin on the formation of BK in plasma activated by dextran sulfate. Plasma was incubated with Neurotropin in the presence of 100 μg/mL (●) or 20 μg/mL (▲) dextran sulfate with 5 mM 1,10-phenanthroline, or 100 μg/mL (○) dextran sulfate without 1,10-phenanthroline. This experiment was performed twice, and a representative experiment is shown. Results in each experiment were virtually identical.

conditions described in Fig. 1. The immunoblots were scanned using a two-dimensional laser densitometer, and the blots and their plots are shown in Fig. 2. Neurotropin inhibited HK cleavage after activation was performed with 20 μg/mL dextran sulfate plus 1,10-phenanthroline as well as with 100 μg/mL dextran sulfate in the absence of 1,10-phenanthroline but did not inhibit the cleavage with 100 μg/mL dextran sulfate plus 1,10-phenanthroline. Therefore, inhibition of HK cleavage paralleled inhibition of BK formation.

Effect of Neurotropin on kallikrein

Since Neurotropin inhibited BK formation and HK cleavage, the simplest explanation would be that it inhibits one of the critical enzymes required for kinin formation, namely, kallikrein. The effect of Neurotropin on the digestion of the synthetic substrate (Chromozym PK) by purified kallikrein was tested. No inhibitory effect of Neurotropin on kallikrein was observed (data not shown). We also tested the ability of Neurotropin to inhibit the cleavage of HK by purified plasma kallikrein. The percent cleavage of HK as assessed by immunoblotting was unaltered by Neurotropin. Thus, no inhibition of kallikrein was evident by either assay. Since Neurotropin inhibited kinin formation and HK cleavage in plasma, we also assessed the evolution of kallikrein activity in plasma by quantitating its stoichiometric interaction with C1 inhibitor to form a complex using the double antibody enzyme-linked immunosorbent assay (ELISA) (Fig. 3). There was prominent inhibition of kallikrein–C1 inhibitor complex formation using 20 μg/mL dextran sulfate plus 1,10-phenanthroline, and lesser inhibition using 100 μg/mL dextran sulfate in the absence of 1,10-phenanthroline and minimal inhibition of 100 μg/mL dextran sulfate plus 1,10-phenanthroline. Again, this result paralleled the inhibition of BK formation or HK cleavage. It was possible, however, that Neurotropin might inhibit the interactions of kallikrein with C1 inhibitor directly. Thus, we added purified kallikrein to normal plasma and determined

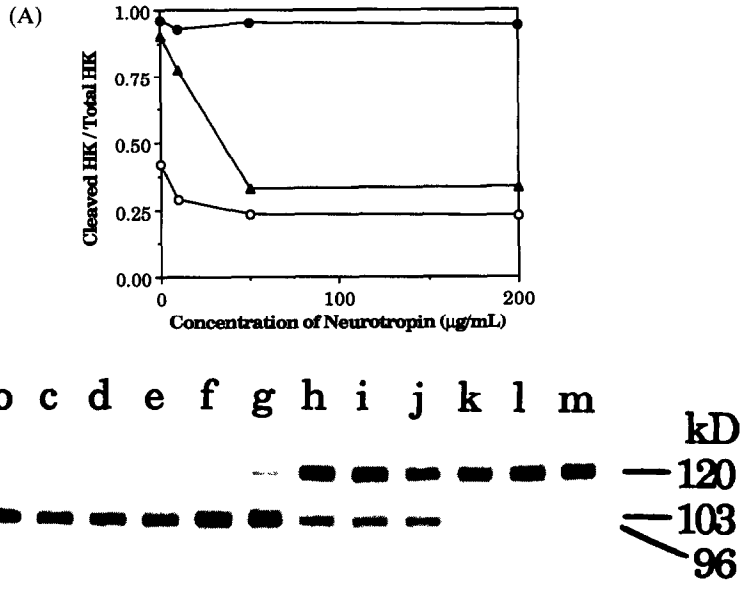

Fig. 2. Effect of Neurotropin on the cleavage of HK in plasma activated by dextran sulfate. (A) Plasma was incubated with Neurotropin in the presence of 100 μg/mL (●) or 20 μg/mL (▲) dextran sulfate with 5 mM 1,10-phenanthroline, or 100 μg/mL (○) dextran sulfate without 1,10-phenanthroline. This experiment was performed twice, and a representative experiment is shown. Results in each experiment were virtually identical. (B) Lanes (a–m) of immunoblots are represented as follows: (a) untreated plasma; (b–e) 100 μg/mL dextran sulfate with 1,10-phenanthroline; (f–i) 20 μg/mL dextran sulfate with 1,10-phenanthroline; and (j–m) 100 μg/mL dextran sulfate without 1,10-phenanthroline. Each sample had 0 (b, f and j), 10 (c, g and k), 50 (d, h and l) and 200 (e, i and m) μg/mL Neurotropin.

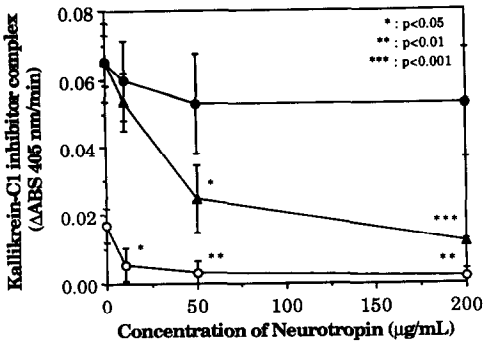

Fig. 3. Effect of Neurotropin on the formation of kallikrein–C1 inhibitor complex in dextran sulfate-activated plasma. Plasma was incubated with Neurotropin in the presence of 100 μg/mL (●) or 20 μg/mL (▲) dextran sulfate with 5 mM 1,10-phenanthroline, or 100 μg/mL (○) dextran sulfate without 1,10-phenanthroline. Each data point represents mean ± SD (N = 4). Statistical analysis was performed using Student's *t*-test.

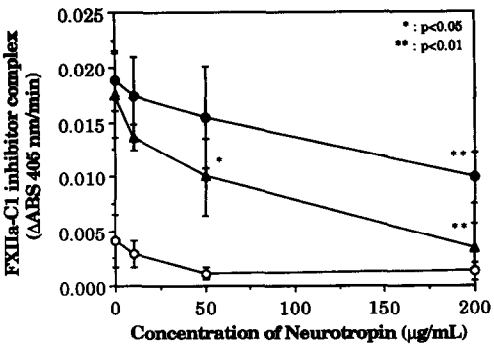

Fig. 4. Effect of Neurotropin on the formation of FXIIa–C1 inhibitor complex in dextran sulfate-activated plasma. Plasma was incubated with Neurotropin in the presence of 100 μg/mL (●) or 20 μg/mL (▲) dextran sulfate with 5 mM 1,10-phenanthroline, or 100 μg/mL (○) dextran sulfate without 1,10-phenanthroline. Each data point represents mean ± SD (N = 4). Statistical analysis was performed using Student's *t*-test.

its rate of complex formation with C1 inhibitor in the presence or absence of Neurotropin. There was no significant effect (data not shown). However, the determination of kallikrein–C1 inhibitor complex levels is a function of the rate of conversion of prekallikrein to kallikrein as well as the interaction of kallikrein with the inhibitor. Since Neurotropin appears to inhibit neither the active enzyme nor complex formation with the inhibitor, the observed effect was most likely due to inhibition of prekallikrein to kallikrein conversion. This may occur either by inhibition of FXII autoactivation, inhibition of FXII activity, inhibition of the interaction of FXIIa with prekallikrein or inhibition of the cofactor function of HK that is required for prekallikrein activation. Therefore, we next assessed each of these possibilities.

Effect of Neurotropin on FXII

We first determined the effect of Neurotropin on the rate of activation of FXII in a plasma system as activated by dextran sulfate and assessed by the rate of formation of FXIIa–C1 inhibitor complex. As shown in Fig. 4, a prominent inhibition of complex formation was seen. We next tried to determine the effect of Neurotropin on FXIIa activity using the synthetic substrate S-2302, but no inhibitory effect was observed in the presence or absence of dextran sulfate (data not shown). We also tested the effect of Neurotropin on FXIIa activity using a natural substrate (prekallikrein) in the presence or absence of 5 μg/mL dextran sulfate. In preliminary studies, we found that dextran sulfate markedly augmented the rate of activation of prekallikrein by FXIIa. Therefore, we utilized a lower concentration of FXIIa in the presence of dextran sulfate so that the rate of activation was similar to the rate seen in the absence of dextran sulfate. Neurotropin had no inhibitory effect on the ability of FXIIa to activate prekallikrein in the absence of dextran sulfate, but a slight and concentration-dependent inhibitory effect (10% inhibition at a 50 μg/mL Neurotropin concentration) could be observed when dextran sulfate was present.

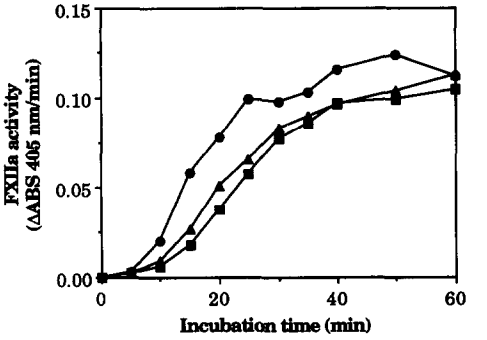

Fig. 5. Effect of Neurotropin on the autoactivation of FXII. FXII (1.25 μM) was incubated with 2.5 nM FXIIa and 5 μg/mL dextran sulfate in the presence of 0 (●), 0.1 (▲) or 1 (■) μg/mL Neurotropin at 37°. Data represent the average of two separate experiments.

Next we assessed the ability of Neurotropin to affect FXII autoactivation by dextran sulfate (Fig. 5). A small, but consistent inhibitory effect of Neurotropin was seen at all time points tested. Since this reaction is exponential [19], we plotted first and second order rate constants for the reaction. In each case, about 26% inhibition was found at 1 μg/mL. Statistical analysis (Student's *t*-test) of second order rate plots (linear portion) revealed a significant difference ($P < 0.05$) between the control and 1 μg/mL Neurotropin. An inhibitory tendency ($0.1 > P > 0.05$) was observed at 0.1 μg/mL Neurotropin. Thus, we observed an inhibitory effect of Neurotropin on FXII autoactivation in a purified system but little effect on FXIIa activity itself, and a prominent effect on FXII activation in plasma as

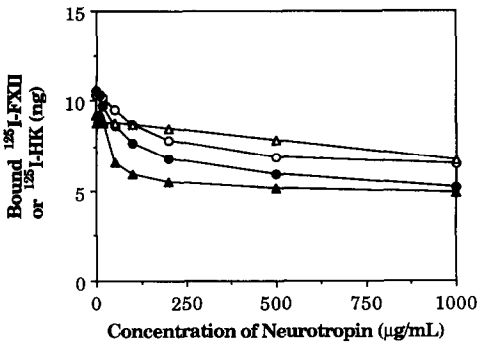

Fig. 6. Effects of Neurotropin on the binding of FXII and HK to kaolin. One milligram of kaolin was incubated with Neurotropin plus ^{125}I-FXII (12.9 ng) or ^{125}I-HK (24.4 ng) in the presence (open symbols) or absence (closed symbols) of unradiolabeled 0.65 μg/mL FXII (○, ●) or 1.6 μg/mL HK (△, ▲). Data are representative of six experiments done with minor variations.

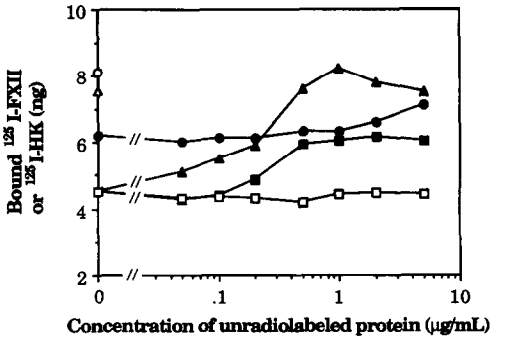

Fig. 7. Effect of unradiolabeled FXII, HK, or the light and heavy chain of HK on the inhibitory action of Neurotropin on the binding of ^{125}I-FXII or ^{125}I-HK to kaolin. The binding of control (no Neurotropin) of ^{125}I-FXII (○) and ^{125}I-HK (△) is also shown. Various concentrations of unradiolabeled FXII (●), HK (▲), or the light (■) and heavy chain (□) of HK were incubated with 1 mg of kaolin plus 100 μg/mL Neurotropin in the presence of ^{125}I-FXII or ^{125}I-HK. Data are representative of two separate experiments.

assessed by complex formation with C1 inhibitor (Fig. 4). Since there is an important feedback system in plasma [20] in which kallikrein cleaves FXII to generate FXIIa, we next examined the effect of Neurotropin on this reaction.

Effect of Neurotropin on the activation of FXII by kallikrein

FXII was incubated with kallikrein plus various concentrations of Neurotropin. These reactions were performed in the presence or absence of dextran sulfate. FXII (2.5 μM) was incubated with 0.25 μM kallikrein plus various concentrations of Neurotropin for 60 min at 37° in the absence of dextran sulfate. No significant autoactivation of FXII should occur in this reaction due to the lack of activating surface. Neurotropin did not inhibit the activation of FXII by kallikrein in the absence of dextran sulfate (data not shown). In a plasma system in which dextran sulfate is present, autoactivation of FXII and activation of FXII by kallikrein are ongoing simultaneously. We therefore next assessed activation of FXII by kallikrein in the presence of dextran sulfate. We first examined the optimal concentration of dextran sulfate required for demonstration of kallikrein cleavage of FXII. A plateau was reached above 10 μg/mL dextran sulfate. We chose 50 μg/mL for subsequent experiments because at this concentration, there was inhibition of FXII autoactivation (optimal concentration of dextran sulfate for autoactivation of FXII was 5 μg/mL). We also limited the time of incubation to 3 min since little autoactivation is seen within this period. The effect of Neurotropin on the activation of FXII by kallikrein in the presence of dextran sulfate was then determined, and again no inhibitory effect was observed.

Effect of Neurotropin on the binding of FXII, FXIIa and HK to the surface and of prekallikrein to HK

Radiolabeled FXII or HK (100,000 cpm) was incubated with kaolin in the presence of various concentrations of Neurotropin. As seen in Fig. 6, there was diminished binding of FXII to kaolin as the concentration of Neurotropin increased. When FXIIa was substituted for FXII, the identical curves were obtained. Since HK also binds to surfaces [21] and such binding is a prerequisite for effective activation of prekallikrein and coagulation factor XI (FXI) [21], we also studied the inhibitory effect of Neurotropin on the binding of HK to kaolin. There was prominent inhibition at a concentration as low as 50 μg/mL (Fig. 6). This inhibition was reversed when an excess of unradiolabeled HK was added. Since kaolin remained in excess, and could bind both labeled and unlabeled kininogen, the reversal was likely due to competition of unradiolabeled kininogen for interaction with Neurotropin. A comparison of this reversal of inhibition by unradiolabeled protein is shown for both FXII and HK in Fig. 7. The reversal appeared more prominent when HK was examined. We also tried to distinguish whether the interaction with heavy chain (HC) or LC derived from cleaved HK. We therefore examined the effect of unradiolabeled, purified LC and HC upon the inhibitory effect of Neurotropin on binding of HK to kaolin. LC reversed the effect of Neurotropin while HC did not (Fig. 7), indicating that the interaction is with LC and confirming that the effect is on the protein rather than the surface. Finally, the order of addition of reagents was examined. If kaolin was incubated with Neurotropin, spun, and then reincubated with protein, no inihibition was seen (Table 1). Thus, Neurotropin does not appear to interact with kaolin to prevent protein binding. If Neurotropin was added after protein binding had occurred, there was no dissociation from the surface. Thus, an interaction with the protein to inhibit binding appears likely.

In separate experiments we examined the effect of Neurotropin upon the binding of prekallikrein to HK. Radiolabeled prekallikrein was incubated with

Table 1. Inhibitory effect of Neurotropin on the binding of FXII and HK to kaolin

Conditions	Inhibitory effect of Neurotropin (%)			
	FXII		HK	
	Concentration of Neurotropin (μg/mL)			
	100	1000	100	1000
Kaolin + Buffer + Neurotropin → mix → spin → + Buffer + Protein → mix → spin	0.6	0	2.5	7.2
Buffer + Neurotropin + Protein → mix → + Kaolin → mix → spin	16.9	37.9	23.3	42.0
Kaolin + Buffer + Neurotropin → mix → + Protein → mix → spin	14.8	60.6	19.9	70.1
Kaolin + Buffer + Protein → mix → + Neurotropin → mix → spin	0.3	0	1.4	1.9
Kaolin + Buffer + Protein → mix → spin → + Buffer + Neurotropin → mix → spin	0	0	5.2	7.0

All inhibition data were obtained by comparing the binding of the sample to its control (no Neurotropin).

HK-coated kaolin in the presence of various concentrations of Neurotropin after blocking the remaining sites on kaolin by 1% non-fat milk. Neurotropin had no inhibitory effect on the binding of prekallikrein. We also incubated Neurotropin with kaolin coated with HK plus prekallikrein in order to test the effect of Neurotropin on the dissociation of prekallikrein from HK, and again no effect of Neurotropin was observed (not shown).

DISCUSSION

Although the mechanism of BK formation in plasma and the tissue kallikrein system have been determined in considerable detail [12, 22, 23], the role of kinin in human diseases has been difficult to assess. The evanescent nature of the active enzymes, and of BK itself, renders them difficult to measure *in vivo*. There are also no specific antagonists that can be employed to inhibit kinin formation in human disease, and thereby indirectly assess the contribution of this pathway to symptoms. Nevertheless, progress has been made and kinin formation has been shown to occur in a wide variety of disorders such as allergic rhinitis [24–27], hereditary angioedema [28–30], pancreatitis [31], infections such as typhoid fever [32], disseminated intravascular coagulation [33, 34], and crystal-induced arthropathies [35]. The specific contribution of BK to the symptoms seen in each these disorders remains uncertain.

In this manuscript we evaluated the ability of Neurotropin to interfere with the activation of the FXII-dependent generation of BK in plasma. The critical initial observation was that contact activation of plasma using dextran sulfate in the presence of Neurotropin leads to inhibition of BK formation (Fig. 1), inhibition of cleavage of HK (Fig. 2) and formation of kallikrein (Fig. 3). Orthophenanthroline was included in the mixture to inhibit kininases and thereby improve the accuracy of the BK determination, and the concentration of dextran sulfate was critical in that the phenomenon could be observed with a low concentration (20 μg/mL) but not with high concentrations such as 100 μg/mL. Some inhibition was observed at 100 μg/mL dextran sulfate if 1,10-phenanthroline was omitted. The difference in the activating capability of dextran sulfate at a low concentration in the presence or absence of 1,10-phenanthroline may be due to the interaction of divalent cations with the K-K cascade.

We then attempted to pinpoint the site of inhibition by Neurotropin and could demonstrate that it is not a kallikrein inhibitor as assessed using a synthetic substrate or by cleavage of HK. It therefore appeared to act on some earlier step, either conversion of prekallikrein to kallikrein or surface-dependent activation of FXII, or both. We next demonstrated that Neurotropin does not diminish the activity of FXII once it is activated or the interaction of prekallikrein with HK, but in plasma it did diminish the rate of conversion of prekallikrein to kallikrein. Although Neurotropin did not inhibit the activation of prekallikrein by FXIIa in the absence of dextran sulfate, it partially inhibited the reaction in the presence of dextran sulfate. Therefore, the effect of Neurotropin appeared to reside at the initiating step of contact activation, i.e. ① autoactivation of FXII to FXIIa, and ② activation of prekallikrein by FXIIa. Neurotropin inhibited FXII autoactivation and had a small inhibitory effect upon the interaction of FXII with a surface such as kaolin. It was not possible to assess this latter effect using dextran sulfate but inhibition of autoactivation by dextran sulfate was observed. There was also prominent inhibition of the binding of HK to kaolin and this

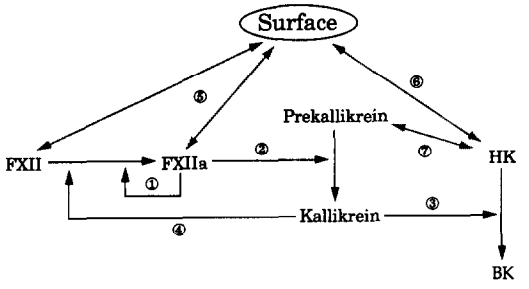

Fig. 8. Possible sites of inhibitory action of Neurotropin on the activation of the kallikrein-kinin cascade and the release of bradykinin. ① the autoactivation of FXII, ② the activation of prekallikrein by FXIIa, ③ the cleavage of HK by kallikrein and release of BK, ④ the activation of FXII by kallikrein, ⑤ the binding of FXII and FXIIa to surfaces, ⑥ the binding of HK to surfaces, and ⑦ the binding of prekallikrein to HK. Neurotropin had a major inhibitory effect on reactions ⑤ and ⑥, and a lesser effect on reactions ① and ② (all involve surface interaction).

effect appeared due to an interaction of Neurotropin with the light chain of HK. Since the interaction of HK with a surface makes an important contribution to the rate of activation of prekallikrein [21], we conclude that these effects are responsible for the diminished conversion of prekallikrein to kallikrein in plasma and the subsequent inhibition of the formation of BK. A summary scheme is shown in Fig. 8. The major inhibitory effects of Neurotropin were on reactions 5 and 6 (interaction of FXII and HK with surface), a lesser effect on reaction 1 (FXII autoactivation-surface initiated), a minimal effect on reaction 2, and no effects on reactions, 3, 4 and 7. The efficiency of Neurotropin as a pain reliever and on permeability reactions seen in allergic disorders may relate to this ability to inhibit BK formation *in vitro*.

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