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Effect of Neurotropin® on the Binding of High Molecular Weight Kininogen and Hageman Factor to Human Umbilical Vein Endothelial Cells and the Autoactivation of Bound Hageman Factor

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ABSTRACT. Bradykinin is generated by activation of the plasma kallikrein-kinin (K-K) cascade and contributes to the symptoms of allergic reactions and the perception of pain. Neurotropin® is a biological material obtained from inflamed rabbit skin inoculated with vaccinia virus, which is widely used clinically in Japan as an effective agent for these disorders. Factor XII (FXII) and high molecular weight kininogen (HK), two critical constituents of the plasma K-K cascade, bind to endothelial cells, and bound FXII is autoactivated in the presence of zinc ions. We have investigated the effects of Neurotropin® on the interactions of FXII and HK with endothelial cells. Neurotropin® inhibited the binding of both proteins to cultured human umbilical vein endothelial cells (HUVEC) and inhibited autoactivation of FXII upon HUVEC in a concentration-dependent manner. 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 bradykinin. BIOCHEM PHARMACOL **55**;8:1175–1180, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. bradykinin; kininogen; neurotropin; factor XII; endothelial cells

We have shown previously that Neurotropin® is capable of inhibiting the interactions among FXII‖, prekallikrein, and HK with negatively charged surfaces, such as kaolin or macromolecular dextran sulfate [1]. Bradykinin formation in plasma is thereby inhibited. We investigated the mechanism(s) of this effect and determined that Neurotropin® inhibits the interaction of HK with negatively charged initiating surfaces and, to a lesser degree, it also inhibits binding of FXII. The effect on HK was shown to be due to an interaction with the LC derived by reduction and alkylation of kinin-free kininogen [1].

Both HK and FXII [2] bind to HUVEC in a zinc-dependent reaction, and competition experiments indicate that they may share a common receptor [3]. Slow activation of FXII occurs upon binding of FXII to the vascular receptor, as assessed by the subsequent conversion of prekallikrein to kallikrein. This contrasts with inert substances such as kaolin, where there is no ion requirement for binding and activation is rapid. In this study, we have examined the effect of Neurotropin® upon the interactions of both HK and FXII with their receptor and upon activation of the cascade. We demonstrated that Neurotropin® inhibits binding of each protein to endothelial cells and that activation of prekallikrein is diminished as a consequence. Neurotropin® may thereby act *in vivo* to inhibit bradykinin formation, further emphasizing the importance of activation along the inner lining of blood vessels.

MATERIALS AND METHODS

Reagents

All chemicals were from the Sigma Chemical Co. unless otherwise specified. Purified proteins, HK, FXII, and prekallikrein were purchased from Enzyme Research Lab. Inc. (4-Amidinophenyl)-methanesulfonylfluoride was from Boehringer Mannheim. The LC of HK and the HC of HK were prepared as published previously [2]. Radiochemical

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‖ *Abbreviations:* FXII, coagulation factor XII; FXIIa, activated coagulation factor XII; HK, high molecular weight kininogen; HUVEC, human umbilical vein endothelial cells; LC, light chain; HC, heavy chain; and gC1qR, receptor for the globular heads of C1q.

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[^{125}I]NaI was obtained from DuPont. Tissue culture 96-well plates were from the Dynatech Co. Medium 199, fetal bovine serum (FBS), and NU-serum were from Life Technologies. A 96-well plate UV reader was obtained from Molecular Device.

Neurotropin® was a gift from the Nippon Zoki Pharmaceutical Co., Ltd. It is an extract of inflamed rabbit skin that has no detectable protein content and specifically lacks any FXII, prekallikrein, or HK. The latter assays included: (1) a specific coagulant assay for each factor using congenitally deficient plasma, (2) conversion of FXII to FXIIa and prekallikrein to kallikrein followed by the assay of each active enzyme utilizing synthetic substrates specific for each, and (3) bradykinin by radioimmunoassay after incubation with exogenous kallikrein so as to digest any HK that might be present. The biological activity of Neurotropin® is determined by an analgesic test in SART (Specific Alteration of Rhythm in environmental Temperature)-stressed animals. Analgesic activity is expressed in Neurotropin® units (U) from concentration–response curves of single administrations. At an intraperitoneal dose of 100 U/kg, Neurotropin® shows 50% analgesic activity [4].

Cell Culture

HUVEC were prepared according to the method of Jaffe [5]. For all experiments, we used the 3rd passage of confluent monolayers of HUVEC within 48 hr. The medium was changed for final passage from M199-containing 20% FBS and 20% Nu-Serum to serum-free medium containing 10 μg/mL of hydrocortisone, 50 μg/mL of bovine pituitary extract, and 50 μg/mL of epidermal growth factor.

Preparation of Radiolabeled Proteins

^{125}I-Labeling of each protein was by the chloramine T method using Iodo-beads™ and [^{125}I]NaI. Over 95% of [^{125}I] proteins were precipitable by the trichloroacetic acid method.

Binding Assay

The monolayers of HUVEC were first washed four times with the binding buffer in the presence of zinc ion (10 mM of HEPES, 11.7 mM of D-glucose, 157 mM of NaCl, 1 mM of $CaCl_2$, 4 mM of KCl, 0.5 mg/mL of BSA, pH 7.40). A 12.4-nM concentration of [^{125}I]FXII or 8.7 nM of either [^{125}I]HK, or [^{125}I]HC of HK, or [^{125}I]LC of HK in binding buffer plus 50 μM of $ZnCl_2$ was added and incubated for 90 min at 37°. After incubation, the cells were washed, and the bound radiolabeled proteins were measured by a γ-counter.

To assess any inhibitory effect of Neurotropin® on the binding of HK or FXII to HUVEC, HUVEC were incubated together with 8.7 nM of ^{125}I-radiolabeled protein and various concentrations of Neurotropin® for 120 min at 37°. Inhibition was expressed as the fraction of bound radioligand in the presence of Neurotropin® divided by bound radioligand in its absence. We also determined whether Neurotropin® could displace HK once it was bound. [^{125}I]HK at 8.7 nM was incubated with HUVEC for 60 min at 37°. Neurotropin® (100 mU/mL) was added, and incubation was continued for an additional 30 and 60 min. Then the percentage of [^{125}I]HK remaining bound was determined.

We next considered the possibility that the inhibitory effect of Neurotropin® might be due to an interaction with HUVEC rather than the protein being studied. For these experiments, we preincubated HUVEC with increasing concentrations of Neurotropin®, from 0 to 100 mU/mL, for 20 min in binding buffer. The supernatant was transferred to another monolayer of HUVEC, both sets were incubated with 8.7 nM [^{125}I]HK, and the binding was quantitated. In a separate experiment, binding was determined after HUVEC were preincubated with 100 mU/mL of Neurotropin® for 0–18 hr in serum-free medium at 37°, and then were incubated with 8.7 nM of [^{125}I]HK for 2 hr at 37° in binding buffer. The effect of Neurotropin® was compared with a different preincubation time prior to the addition of HK.

Activation of FXII

We modified the method of Silverberg and Diehl [6] to quantitate activation of FXII. All reactions were carried out at 37° in a polystyrene microtiter 96-well plate that had been coated for 2 hr with the reaction buffer consisting of 10 mM of HEPES, 137 mM of NaCl, 11 mM of D-glucose, 4 mM of KCl, and 0.5 mg/mL of BSA (pH 7.40). FXII and prekallikrein were dissolved at 1.45 and 1.38 mg/mL, respectively, in the stock buffer consisting of 10 mM of sodium acetate, 150 mM of NaCl, and 0.01% Triton X-100 (pH 5.0), and were stored at −70° after treatment with (4-amidinophenyl)-methanesulfonylfluoride to remove traces of FXIIa and kallikrein. Before use, both proteins were thawed and retreated with (4-amidinophenyl)-methanesulfonylfluoride for 1 hr and diluted to 10 μg/mL in reaction buffer. To quantitate activation of FXII by HUVEC, 10 μL of 124-nM FXII was added to a monolayer of HUVEC (96-well plate) plus 70 μL of reaction buffer and preincubated in the absence of zinc ions. Ten microliters of 125-nM prekallikrein and 10 μL of 6-mM *N*-benzoyl-L-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide acetate (chromozym PK) were added, and kallikrein was determined by the evaluation of *p*-nitroaniline. A second set was assayed in which the initial incubation of FXII included zinc ions. Then the cells were washed with the binding buffer containing zinc ions. Eighty microliters of reaction buffer, 10 μL of 125-nM prekallikrein, and 10 μL of 6-mM chromozym PK were added. Kallikrein was monitored by the increase in the absorbance of chromogenic substrate hydrolysis and was measured by absorbance

changes at 405 nm with a microplate reader THERMOmax™ thermostated at 37°.

To determine the effect of Neurotropin® on the ability of FXIIa to convert prekallikrein to kallikrein, HUVEC were incubated with 12.4 nM of FXII for 120 min at 37°; then 12.5 nM of prekallikrein and 0–100 mU/mL Neurotropin® were added, and kallikrein activity was determined.

RESULTS

We first studied the ability of Neurotropin® to inhibit the binding of ^{125}I-labeled HK, HC-HK, or LC-HK to HUVEC in the presence of increasing concentrations of Neurotropin® for 2 hr at 37°. As shown in Fig. 1A, Neurotropin® inhibited the binding of HK in a concentration-dependent fashion that was optimal between 50 and 100 mU/mL. Approximately 80–90% inhibition was achieved. When HC and LC binding were similarly assessed, there was comparable inhibition of LC binding to HUVEC (Fig. 1C). HC demonstrated less total binding under identical binding conditions, and was inhibited minimally (<20%), as shown in Fig. 1B. The IC_{50} values for HK and LC-HK were 25 and 35 μg/mL, respectively.

Because we previously demonstrated that FXII also binds to HUVEC in a zinc-dependent fashion, and may utilize the same receptor site as does HK [3], we next examined the ability of Neurotropin® to inhibit FXII binding. As shown in Fig. 2, inhibition was also evident between 10 and 100 mU/mL, and 50% inhibition was achieved at 60 mU/mL. Thus, the effects of Neurotropin® upon HK and FXII binding to HUVEC are strikingly similar.

FXII is known to autoactivate upon initiating surfaces [7], and we have shown that binding to endothelial cells slowly catalyzes conversion to FXIIa under conditions in which measurable FXIIa is not seen in the fluid phase [3]. The assay utilizes conversion of prekallikrein to kallikrein as an assay of FXIIa activity. In Fig. 3, we incubated FXII plus HUVEC for 30 min in the presence of increasing concentrations of Neurotropin®, as indicated. Then prekallikrein was added, and the effect of Neurotropin® upon kallikrein activity was determined. Inhibition was seen between 1 and 100 mU/mL, presumably reflecting the inhibition of binding seen in Fig. 2. To assess whether there is either inhibition of FXII autoactivation or inhibition of the ability for FXIIa to convert prekallikrein to kallikrein, we first incubated FXII alone with HUVEC for 120 min, then prekallikrein and Neurotropin® (0–100 mU/mL) were added sequentially, and the rate of conversion to kallikrein was determined. As shown in Fig. 4, Neurotropin® had no effect. Thus, Neurotropin® does not affect the activation of prekallikrein by FXIIa.

Next, we studied the displacement of [^{125}I]HK from HUVEC by Neurotropin®, which was added after 60 min of incubation time. As shown in Fig. 5, Neurotropin® at 100 mU/mL displaced bound HK at 120 min from 6.8 ± 0.5 to 3.6 ± 0.3 pmol/10^6 cells. Thus, Neurotropin® appears more effective in preventing binding than in reversal of binding

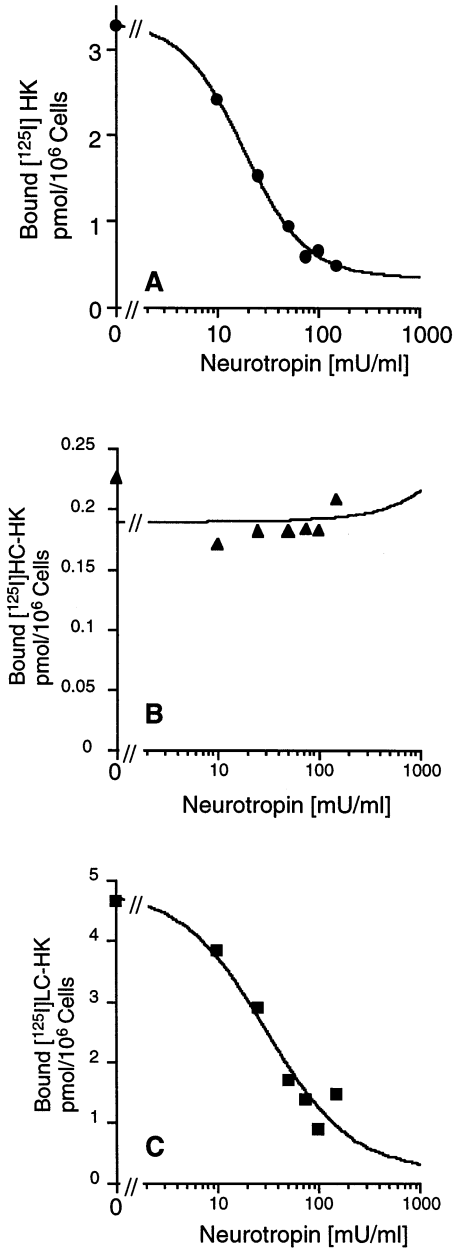

FIG. 1. Inhibition of HK (A), HC (B), and LC (C) by Neurotropin®. ^{125}I-Labeled HK (8.7 nM) or purified HC or LC of HK were incubated with a monolayer of HUVEC in the presence of increasing concentrations of Neurotropin® plus 50 μM of $ZnCl_2$ for 120 min at 37°. The binding of ^{125}I-labeled proteins was then related to the concentration of Neurotropin®. The experiment was performed four times, and a representative experiment is shown.

that is already established. Nevertheless, reversal of binding is possible.

We next studied the effect of preincubation of Neurotropin® with HUVEC upon subsequent binding of HK. Prior studies using kaolin suggested that Neurotropin® interacted with HK rather than with the kaolin surface [1]. The human receptor is presumably protein, and interaction with it is also possible. HUVEC were therefore preincubated with increasing concentrations of Neurotropin® for

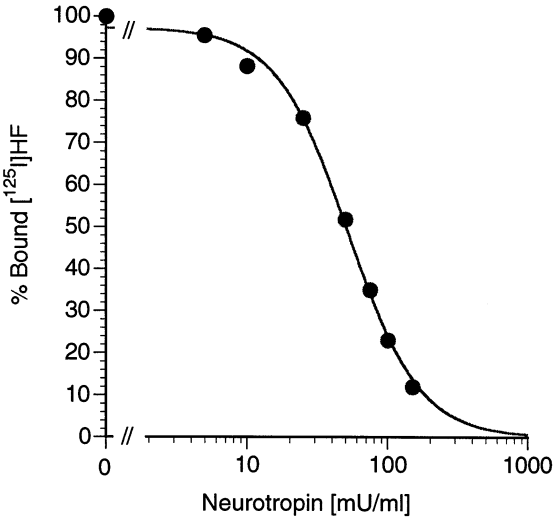

FIG. 2. Inhibition of FXII (HF) binding to HUVEC by Neurotropin®. ^{125}I-Labeled FXII (12.4 nM) was incubated with a monolayer of HUVEC in the presence of increasing concentrations of Neurotropin® plus 50 μM of $ZnCl_2$ for 120 min at 37°, and the percentage of bound protein was determined. The figure is representative of four experiments with similar results.

20 min at 37° in the presence of 50 μM of $ZnCl_2$, and the supernatant was reincubated with fresh HUVEC plus [^{125}I]HK for 2 hr at 37°. The HUVEC exposed to Neurotropin® were washed with binding buffer contained 50 μM of $ZnCl_2$, and then were incubated with [^{125}I]HK for 2 hr at 37°. The inhibitory activity of Neurotropin® was transferred with the supernatant, as indicated in Fig. 6A. The

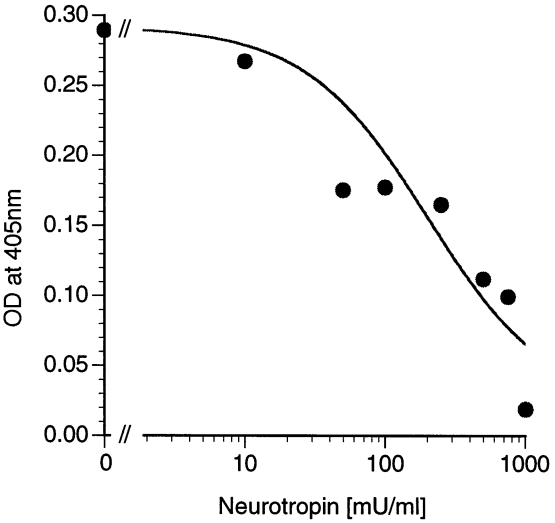

FIG. 3. Inhibition of FXII-dependent prekallikrein activation by Neurotropin®. FXII (12.4 nM) was preincubated with a monolayer of HUVEC in the presence of increasing concentrations of Neurotropin® plus 50 μM of $ZnCl_2$ for 30 min at 37°. The unbound FXII and Neurotropin® were removed, and the cells were washed and reincubated with 12.5 nM of (4-amidinophenyl)-methanesulfonylfluoride (APMSF)-treated prekallikrein and 0.6 mM of chromozym PK. Kallikrein activity was measured by O.D. at 405 nm. The figure is representative of four experiments with similar results.

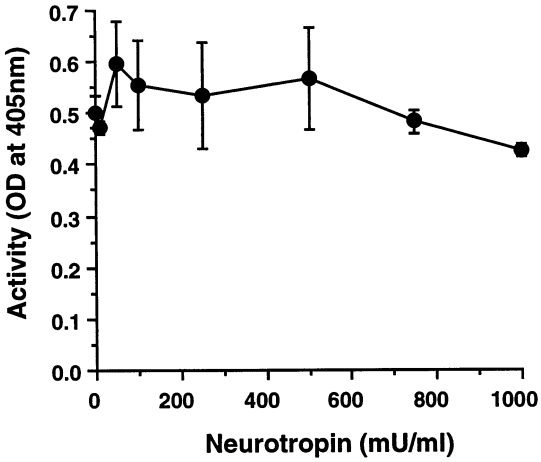

FIG. 4. Effect of Neurotropin® upon activation of prekallikrein by FXIIa. FXII (12.4 nM) was preincubated with a monolayer of HUVEC in the presence of 50 μM of $ZnCl_2$ for 120 min at 37°, the unbound FXII was removed, and the cells were washed. Then HUVEC were incubated with 12.5 nM of APMSF-treated prekallikrein plus 0.6 mM of chromozym PK in the presence of increasing concentrations of Neurotropin®. Values are means ± SEM for four experiments.

HUVEC exposed to Neurotropin® bound the same amount of HK regardless of the concentration of Neurotropin® used, and extending the preincubation time up to 18 hr did not affect binding of [^{125}I]HK to HUVEC (Fig. 6B).

DISCUSSION

Bradykinin is a vasoactive peptide that causes vasodilatation, increases vascular permeability, induces a burning-

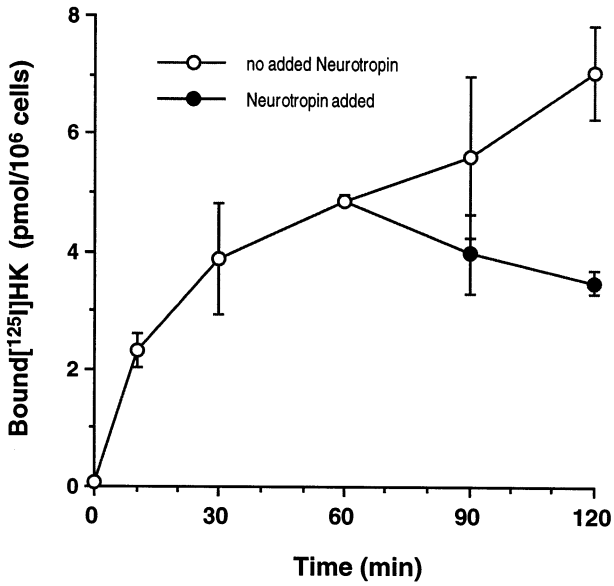

FIG. 5. Reversal of HK binding by Neurotropin®. ^{125}I-Labeled HK (8.7 nM) was incubated with a monolayer of HUVEC in the presence of 50 μM of $ZnCl_2$ at 37°. Neurotropin® (100 mU/mL) was added to the incubated cells at the 60-min time point, and then incubated for an additional 30 and 60 min. The binding of ^{125}I-labeled HK was determined. Values are means ± SEM for four experiments.

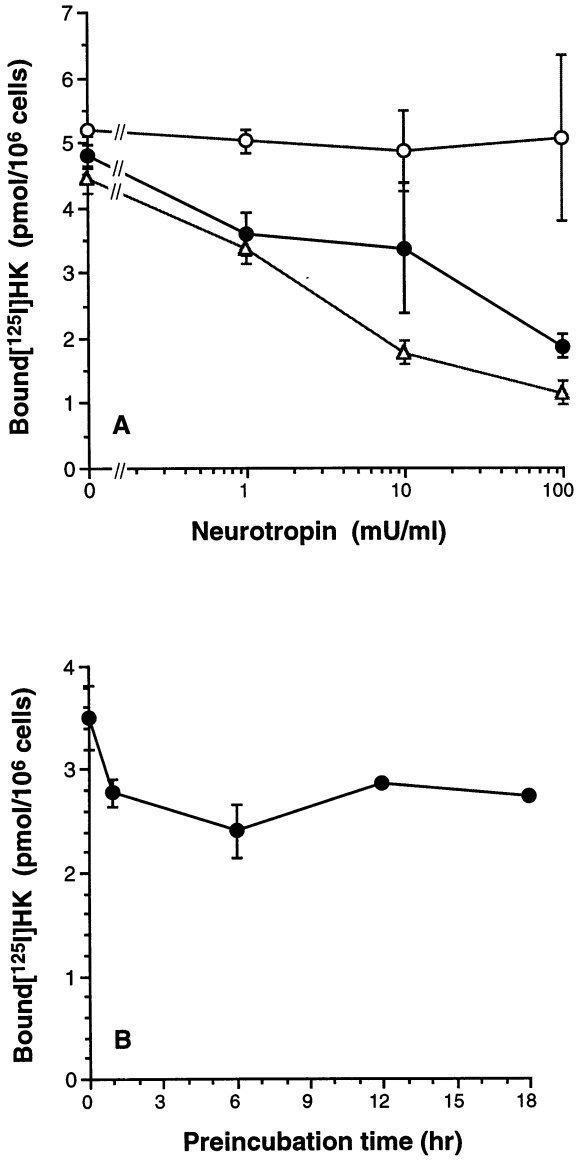

FIG. 6. Effect of Neurotropin® on HUVEC or HK. (A) [^{125}I]HK (8.7 nM) was incubated with HUVEC in the presence of increasing concentrations of Neurotropin®, and binding was determined (●). In addition, HUVEC were incubated with increasing concentrations of Neurotropin®. The supernatant was removed, the cells were washed, and then they were incubated with [^{125}I]HK and binding was determined (○). The supernatant was added to a fresh lot of HUVEC and incubated together with [^{125}I]HK, and binding again was determined (△). (B) Preincubation of HUVEC with Neurotropin® for up to 18 hr followed by washing and then assessment of [^{125}I]HK binding. Values are means ± SEM for four experiments.

type pain upon contact with sensory nerve endings, and, depending upon the tissue, activates phospholipases and the various cascades dependent upon arachidonic acid mobilization. Most of these effects are mediated by a B_2 receptor and one of the primary sites of such receptors is vascular endothelial cells [8–11].

The formation of bradykinin in plasma is dependent on the interactions of FXII (Hageman factor), prekallikrein, and HK and is controlled by inhibitors such as C1 inactivator and α_2-macroglobulin. Recent data have demonstrated that FXII and HK can bind to HUVEC [12, 13], and FXII and HK may interact with the same receptor [3]. Recent evidence suggests that this binding protein is identical to the receptor for the globular heads of C1q component of complement (gC1qR) [14, 15]. Prekallikrein is present by virtue of its binding to a specific domain on the LC of HK [16]. Thus, all of the factors required for contact activation-dependent kinin formation can be assembled along this endothelial cell surface, presumably in proximity to the bradykinin receptor itself [17]. Neurotropin® also has anticomplementary activity *in vivo* [18, 19]. FXII fragment activates C1 [20], and gC1qR modulates the complement pathway after C1q binds to it [21, 22]. However, the effect of Neurotropin® on the binding of C1q to gC1qR is unclear, and neither FXII nor HK competes with C1q binding to gC1qR [14].

We have shown previously that Neurotropin® is capable of inhibiting the formation of bradykinin when activation is examined in the fluid phase using initiators such as kaolin in dextran sulfate. However, the interactions along the vessel wall are far more likely to be physiologic, and there is evidence that slow activation of the cascade may be a result of binding [3]. Thus, we have examined the ability of Neurotropin® to inhibit the interactions of these proteins with the endothelial cell receptor and with each other. The data demonstrate inhibition of binding and activation of FXII and HK by Neurotropin® at concentrations that are also active in the fluid phase and suggest interaction with the LC of kininogen as well as with FXII. We had no evidence of binding to the cell surface receptor (presumably gC1qR). These interactions inhibit the initiation of contact activation. Activation of FXII upon the surface is inhibited, while conversion of prekallikrein to kallikrein is not inhibited by Neurotropin® if FXIIa is already formed. The data further suggest that it is more difficult to reverse binding than it is to prevent binding by prior interaction with plasma proteins. However, partial reversal is seen. Although little is known about HK or FXII turnover along the endothelial cell surface, we would anticipate gradual diminution of the amount bound if a steady-state concentration of Neurotropin® in plasma were established. This drug is currently utilized as therapy for allergic reactions and to alleviate pain in rheumatic conditions [23]; however, its mechanism of action is unclear. Our data suggest that it impedes the generation of bradykinin and, therefore, may represent the first agent whose action is attributable to this mechanism.

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