Identification of Tissue Kallikrein in Brain and in the Cell-free Translation Product Encoded by Brain mRNA*

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A monoclonal antibody against purified rat urinary kallikrein was coupled to agarose and used to isolate kallikrein from rat brain. The purified enzyme has N\(^{-}\)-tosyl-L-arginine methyl esterase activity with a pH optimum at 9.0, kinin-releasing activity from a purified low molecular weight kinogen, and a parallelism with standard curves of rat urinary kallikrein in a direct radioimmunoassay. Brain kallikrein is inhibited by a series of tissue kallikrein inhibitors with IC\(_{50}\) values similar to those for urinary kallikrein. The purified brain enzyme was labeled with \(^{14}C\)diisopropylphosphorofluoridate and visualized by fluorography on a sodium dodecyl sulfate-polyacrylamide gel. Electrophoretic mobility of the enzyme was closely similar to that of urinary kallikrein with estimated \(M_\text{r}\) of 38,000. With Western blot analyses using a rabbit anti-kallikrein antibody, both brain and urinary kallikrein were visualized at identical positions by immunoperoxidase staining and by autoradiography with \(^{125}\)I-protein A binding. Brain mRNA was used to direct cell-free protein synthesis in wheat germ and rabbit reticulocyte lysate systems. \(^{35}\)S Methionine-labeled kallikrein was identified by fluorography of sodium dodecyl sulfate-polyacrylamide gels after the translation products were subject to immunoprecipitation with affinity-purified kallikrein antibody. Collectively, the data show that tissue kallikrein exists in brain and can be synthesized by brain mRNA.

Tissue kallikrein (EC 3.4.21.8) is being found in sites not previously considered to contain this activity, such as plasma, vascular endothelium and thyroid (1–5). Whether a tissue kallikrein is actually present in the brain has been uncertain but some kinin-generating activity was detected in homogenates of rabbit (6, 7) and rat (8, 9) brain. Highest activities were noted in cerebral cortex with less in brainstem and cerebellum (8). Recently, a kinin-forming enzyme activity with some similarity to tissue kallikrein was also found in rat and porcine anterior pituitary gland (10, 11). This activity differs from tissue (renal) kallikrein with respect to susceptibility to inhibitors and substrate specificity.

The central nervous system administration of the kinin

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†† The abbreviations used are: MOPS, 4-morpholinopropanesulfonic acid; TosArgOMe, \(N^\text{-tosyl-L-arginine methyl ester}; SBTI, soybean trypsin inhibitor; LBTI, lima bean trypsin inhibitor; OMTI, ovomucoid trypsin inhibitor; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; Heps, 4-(2-hydroxyethyl)-1-piperazin-ethanesulfonic acid.

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Peritoneally with 1 were dispersed into multiwell dishes in a selective medium supplemented with 0.2 ml of Pristane for 2 weeks, then injected intraperitoneally with 0.2 ml of pristane for 2 weeks. The supernatant was centrifuged at 105,000 x g for 20 min, and the supernatant was resuspended in the buffer and centrifuged at 105,000 x g for 20 min. The first 10,000 x g supernatant was centrifuged for 20 min at 105,000 x g in a Beckman L5-65 ultracentrifuge. The 105,000 x g supernatant was centrifuged at 27,000 x g for 15 min. The pellet containing RNA from the 27,000 x g fraction was resuspended in 60 ml of the same buffer, and sodium deoxycholate and Triton X-100 were added at final concentrations of 1% each. The suspension was centrifuged at 10,000 x g for 10 min. RNA was extracted from the supernatant by phenol-chloroform-isomyl alcohol treatment (33). Polyadenylate-containing mRNA was isolated by oligo(dT)-cellulose chromatography (34).

Isolation of Poly(A) mRNA from Brain Approximately 80 g of rat whole brain tissue were minced and homogenized at 4 °C with a Polytron instrument in 5 volumes of buffer (25 mM Tris/His, pH 7.5, 0.25 mM sucrose, 25 mM NaCl, 5 mM MgCl2, 0.1% diethylpyrocarbonate, and 0.1 mM cycloheximide). The homogenate was centrifuged at 1000 x g for 10 min, and the supernatant was further centrifuged at 27,000 x g for 15 min. The protocol was performed at 10 °C. The reaction was terminated after 20 min by washing with H2O.

(a) Immunoperoxidase Staining Method—Kallikreins were visualized by immunoperoxidase staining as described by Towbin et al. (31). After electrophoresis, the gels were stained for 30 min in 1% 3,3'-diaminobenzidine (DAB) containing 1% hydrogen peroxide. The gels were washed 3-4 times with buffer A. Total and immunoprecipitated translation products were analyzed by SDS-polyacrylamide gel electrophoresis and radioactive protein bands were visualized by fluorography as described above.

MATERIALS

The following materials were obtained from commercial sources: molecular weight markers, protein A and protein A-Sepharose (Pharmacia); enzyme-grade ammonium sulfate, ultrapure sucrose, TosArgOMe, bovine serum albumin, bradykinin, crystalline ovalbumin, chymotrypsinogen A (Schwarz/Mann); TosArgO[7H]Me (Amersham Corp.); [14C]diisopropylphosphofluoridate (specific activity, >100 Ci/mmol), ENHANCE (New England Nuclear); polyacrylamide gel electrophoresis and radioactive protein bands were visualized by fluorography as described above.

Western Blot Analyses

(a) Immunoperoxidase Staining Method—Kallikreins were visualized by immunoperoxidase staining as described by Towbin et al. (31). After electrophoresis, the gels were stained in 0.1% methanolic acid-water (4:5:45) containing 2.5% Coomassie blue and destained overnight in methanol-acetic acid-water. The gels were treated with ENHANCE and then dried on Whatman No. 3MM filter paper. Fluorography was carried out by placing the gels in direct contact with Kodak X-Omat AR film. Films were developed after 1 week of exposure.

(b) Autoradiography with [125I]-Protein A Binding—Alternatively, following incubation of the blot with antibody-antibody kallikrein antibody, the blot was washed and incubated with [125I]-protein A (106 cpn/ml) for 2 h at room temperature. The solution was then aspirated and the nitrocellulose sheet was rinsed with H2O, then soaked with shaking at 10 °C in a buffer of 0.15 M Tris/HCl, pH 7.4, 0.005 M EDTA, 0.15 M NaCl, 0.25% gelatin, 0.5% Triton X-100, and 0.1% SDS at room temperature for 2 h. The sheet was affixed to a sheet of filter paper and exposed to Kodak X-Omat AR film at -70 °C using Cronex lightning plus a intensifying screen for 6-24 h.

Cell-Free Translation Wheat germ extract or rabbit reticulocyte lysate was prepared and used as described previously (35, 36). The reaction mixture (total volume, 50 µl) contained 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate, pH 7.6, 1.5 mM magnesium acetate, 64 mM potassium acetate, 2 mM ATP, 0.1 mM GTP, 10 mM creatine phosphate, 10 µg/ml of creatine phosphokinase, 0.7 mM spermidine, 20-30 µCi of 35S]methionine (specific activity, >100 Ci/mmol), 60% of RNA, 15 µl of wheat germ extract or 30 µg/ml of RNA with reticulocyte lysate, and 15 µl of wheat germ extract or 10 µl of reticulocyte lysate. After incubation at 25 °C for 120 min, with occasional shaking, trichloroacetic acid-insoluble radioactivity was determined. The reaction mixture for protein synthesis was scaled up to 15-fold, and the reaction was terminated by placing the samples on ice. The sample was diluted in 3 volumes of buffer A containing 10 mM Na phosphate, pH 7.5, and incubated for 2 h at room temperature. The reaction was terminated after 20 min by washing with H2O.

Active Site Labeling Rat brain enzyme (10 µg) or purified urinary kallikrein (1 µg) was incubated with 1 Ci diisopropylphosphofluoridate (0.2 µCi; specific activity, >100 µCi/mmol) in a total volume of 30 µl of H2O for 1 h at 37 °C followed by 16 h at 4 °C. The mixture was then added to 30 µl of buffer containing 0.13 M Tris/HCl, pH 6.8, 30% glycerol, 10% mercaptoethanol, and 5% SDS and heated to 100 °C for 5 min. Electrophoresis was performed in a 7.5 to 17.5% linear gradient polyacrylamide gel according to the procedures described previously (25, 30). After electrophoresis, the gels were stained in 0.1% Coomassie blue and destained overnight in methanol-acetic acid-water. The gels were treated with ENHANCE and then dried on Whatman No. 3MM filter paper. Fluorography was carried out by placing the gels in direct contact with Kodak X-Omat AR film. Films were developed after 1 week of exposure.

Materials

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(Eastman); specially pure SDS (BHA Chemicals); human α1-antitrypsin (Sigma); SBTI, OMTI, LBTI (Worthington); Affi-Gel 10 and 15 (Bio-Rad); complete Freund's adjuvant (Difco); Hepes (Calbiochem-Behring); Nonidet P-40 (Bethesda Research Laboratories); peroxidase-conjugated rabbit anti-mouse IgG, peroxidase-conjugated goat anti-rabbit IgG; rabbit antinouse IgG, IgGα, IgGβ, IgGγ, IgM (Cappel Laboratories); rabbit anti-mouse IgG (Sigma); Pristane (2,6,10,14-tetramethylpentadecane) (Aldrich); antibiotic-antimycotic solution, fetal calf serum, Dulbecco's modified Eagle's medium (Gibco); nitrocellulose paper BA85, 0.45 μm, (Schleicher & Schuell); and microtiter ELISA plates (Dynatech). Aprotinin was a gift from Dr. G. L. Haberland, Bayer A. G., Wuppertal-Elberfeld, West Germany; D-PhePheArgCH2Cl was a gift from Drs. C. Kettner and E. Shaw, Brookhaven National Laboratory; pentamidine was a gift from Drs. J. D. Geratz and R. R. Tidwell, University of North Carolina; antipain and leupeptin were provided by the United States-Japan Cooperative Cancer Research Program. Purified bovine low molecular weight kininogen was a gift from Drs. S. Iwanaga and H. Kató of the Protein Research Institute, University of Osaka, Osaka, Japan.

RESULTS

Specificity of Monoclonal Antibody—The monoclonal antibody secreted by the hybridoma clone designated V4Gs was harvested from culture media or ascites fluid as described under “Experimental Procedures.” The antibody titration curves of Fig. 1 show that the polyclonal antibody at a final dilution of 1:4 x 10^3 not only binds kallikrein but also purified arginine esterase A (20), while the monoclonal antibody binds only to kallikrein over a broad range of dilution. This monoclonal antibody was, therefore, used to prepare an antibody-affinity column for brain kallikrein purification.

Purification of Brain Tissue Kallikrein—Kallikrein-like activity was measured by the TosArgOMe esterase radiochemical method. Rat brain (-40 g wet tissue at a time) was homogenized and centrifuged as described under “Experimental Procedures.” After dialysis and DEAE-cellulose column chromatography as previously described (19–21), TosArgOMe esterase activity binding to the column was eluted between NaCl concentrations of 0.2–0.3 M. The fractions containing activity were pooled and adjusted to 0.2 M NaCl, 0.01 M NaH2PO4, pH 6.0. The pooled fractions were applied to the monoclonal antibody affinity column (1.5 x 15 cm) equilibrated with the same buffer. As shown in Fig. 2, most of the protein flowed through the column and the adsorbed TosArgOMe esterase activity was eluted with 0.1 M acetate buffer, pH 3.5.

Brain Kallikrein Assays and Characteristics—Appropriate

FIG. 1. Monoclonal antibody-affinity chromatography of rat brain esterase. Column, 1.5 x 15 cm; starting material, 150 A280 units of protein from a DEAE-cellulose column in 200 ml of 0.2 M NaCl, 0.01 M Na phosphate, pH 6.0; fractions 1–34, volume 9 ml; fractions 35–50, volume 5 ml; flow rate 40 ml/h.

FIG. 2. Kallikrein antibody titration curves. Polyclonal antirat urinary kallikrein antiserum or monoclonal antibody against rat urinary kallikrein produced in ascites fluid of BALB/C mice were diluted to the indicated final concentrations. One-tenth ml of the antibody was incubated with 0.1 ml of either 125I-rat urinary kallikrein or 125I-rat urinary esterase A (20) in a final volume 0.4 ml at 4°C for 24 h. Antibody-bound tracer was separated from the free form with a polyethylene glycol separation technique. ○, ▲, 125I-rat urinary kallikrein; ○, Δ, 125I-rat urinary esterase A. ○, ●, polyclonal antiserum; ▲, △, monoclonal antibody.

FIG. 3. Log-logit transformation of a typical (radioimmunoassay standard curve of rat urinary kallikrein (○—○) and rat brain kallikrein (▲—▲). B/B0 is the per cent of bound radioactivity in the presence and absence of unlabeled kallikrein using a sheep anti-rat urinary kallikrein antiserum (26).
dilutions of the affinity column eluate were used to obtain a range of antigen concentrations comparable to the rat urinary kallikrein standard (0.08-20 ng/tube). Fig. 3 shows typical log-logit transformations of radioimmunoassay standard curves of rat urinary kallikrein and serial dilutions of the rat brain esterase. Linear curves which are always parallel to the curves of rat urinary kallikrein and serial dilutions of the rat brain esterase have these enzymatic qualities indistinguishable from those of rat urinary kallikrein.

The pH profile of TosArgOMe esterase activity of the rat brain enzyme had TosArgOMe esterase activity of 116 e.u./mg of enzyme as calculated from the direct radioimmunoassay. The enzyme also released kinin from purified bovine low molecular weight kininogen substrate at a rate of 3.3 μg of kinin generated/min/mg of kallikrein. Thus, the rat brain esterase has these enzymatic qualities indistinguishable from those of rat urinary kallikrein.

The pH profile of TosArgOMe esterase activity of the rat brain enzyme followed a Gaussian distribution with optima of 9.0, similar to rat urinary (renal) kallikrein. A comparison of the effects of various serine proteinase inhibitors on rat brain and urinary kallikrein activities is shown in Table I.

**Table I**

| Inhibitor                  | 1C50, Rat brain kallikrein | 1C50, Rat urinary kallikrein |
|----------------------------|----------------------------|----------------------------|
| Antipain                   | 6.8 × 10^{-5} M            | 6.5 × 10^{-5} M            |
| Leupeptin                  | 5.2 × 10^{-5} M            | 6.9 × 10^{-5} M            |
| PhePheArgCHCl               | 5.2 × 10^{-5} M            | 4.8 × 10^{-5} M            |
| Pentamidine                | 6.5 × 10^{-5} M            | 4.8 × 10^{-5} M            |
| Benzamidine                | 1.5 × 10^{-5} M            | 1.5 × 10^{-4} M            |
| Aprotinin                  | 1.5 units/ml               | 1.5 units/ml               |
| LBTI (170 μg/ml)           | No inhibition              | No inhibition              |
| α1-antitrypsin (170 μg/ml) | No inhibition              | No inhibition              |
| SBTI (180 μg/ml)           | 170 μg/ml                  | 170 μg/ml                  |

**Fig. 4.** SDS-polyacrylamide gel electrophoresis of urinary kallikrein and brain kallikrein. Left, active site labeling of urinary kallikrein and brain kallikrein. The enzymes were incubated with [14C]diisopropylphosphorofluoridate and electrophoresis was performed in a 7.5 to 17.5% linear gradient gel. The protein bands were visualized by fluorography. Molecular weight markers are phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). 1, rat urinary kallikrein B; 2, rat brain kallikrein. Right, Western blot analysis of urinary kallikrein and brain kallikrein. Electrophoresis was performed in a 7.5 to 17.5% linear gradient gel containing 0.1% SDS and proteins were electrophoretically transferred to nitrocellulose paper. The blots were incubated with rabbit anti-rat urinary kallikrein antiserum followed by goat anti-rabbit IgG-horseradish peroxidase conjugate or [125I]-protein A. Horseradish peroxidase conjugate was visualized with a color reaction and [125I]-protein A binding was visualized with autoradiography. A, immunoperoxidase staining; B, [125I]-protein A binding. 1, rat urinary kallikrein B; 2, rat brain kallikrein.

**Fig. 5.** Electrophoretic analyses of rat brain mRNA-dependent lysate translation products. A, wheat germ system; B, rabbit reticulocyte lysate system. Experimental details are described in the text. Lane 1, control reaction containing no mRNA (~50,000 cpm); lane 2, control immune precipitate from affinity-purified sheep anti-rat urinary kallikrein; lane 3, polypeptides from mRNA-directed translation (~100,000 cpm); lane 4, translation products in sheep anti-rat urinary kallikrein immune precipitate; lane 5, [125I]-urinary kallikrein.
rat urinary kallikrein (19), the rat brain enzyme is inhibited by high concentrations, but not by low concentrations of SBTI.

Active Site Labeling—Purified brain and urinary kallikreins were prelabeled with [14C]diisopropylphosphorofluoridate and then electrophoresed in a polyacrylamide slab gel under reducing conditions. The fluorogram of brain enzyme shows a single band with electrophoretic mobility similar to but slightly slower than that of urinary kallikrein B (Fig. 4, left). The molecular weight of the brain enzyme is approximately 38,000. The data show that the brain enzyme is a serine proteinase as it covalently binds [14C]diisopropylphosphorofluoridate.

Western Blot Analysis—To demonstrate specific recognition by tissue kallikrein antibody, brain kallikrein was subject to SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose paper. After incubation with rabbit anti-kallikrein antibody, both brain and urinary kallikreins were visualized by immunoperoxidase staining (Fig. 4, right, A) or by autoradiography using 125I-protein A binding (Fig. 4, right, B). These Western blot studies show that the brain and urinary enzymes are closely related, that brain kallikrein is recognized by antibody to urinary kallikrein, and are consistent with the results obtained from active site labeling.

Cell-free Translation of Rat Brain mRNA—Poly(A) mRNA was obtained from rat brain after chromatography on oligo(dT)-cellulose and the A260/A280 quotient of the mRNA was 2.10. On incubation of the mRNA in the wheat germ system, 2% of [35S]methionine radioactivity was incorporated into trichloroacetic acid-precipitable protein. This maximal incorporation occurred at an mRNA concentration of approximately 60 μg/ml. Slab gel electrophoresis of rat brain mRNA translation products derived from both wheat germ extract (Fig. 5A) and rabbit reticulocyte lysate (Fig. 5B) is shown. Total translation products or immunoprecipitates in the absence of added brain mRNA were used as controls (lanes 1 and 2). Brain mRNA-directed total products and the product immunoprecipitated by the affinity-purified kallikrein antibody are shown in lanes 3 and 4. The electrophoretic mobilities of kallikrein synthesized in the cell-free systems and immunoprecipitated by the kallikrein antibody are closely similar to that of authentic tissue kallikrein (lane 5).

DISCUSSION

The results of this study demonstrate that tissue kallikrein is present in rat brain. The rat brain enzyme was isolated from deoxycholate-treated brain extracts followed by DEAE-cellulose and monoclonal antibody-affinity column chromatography. The brain enzyme is a serine proteinase, as it is covalently labeled by 14C]diisopropylphosphorofluoridate. Furthermore, the enzyme was isolated in an active form with both TosArgOMe esterase and kininogenase activities. Brain and urinary kallikreins were indistinguishable with respect to molecular weight, immunological properties, pH optimum and susceptibility to inhibitors. Western blot analyses showed that brain kallikrein is specifically recognized by antibody to a purified tissue (urinary) kallikrein. In cell-free translation systems using brain mRNA, labeled kallikrein was identified after specific immunoprecipitation.

Other investigators have explored the presence of components of the kallikrein-kinin system in brain. Hori (7) was the first to suggest the presence of kinin-releasing, and destroying, activities and kinin-like peptides in the rabbit brain. However, acetone or acid treatment was required to elicit what Hori considered only slight kinin-forming activity in brain subcellular fractions. Shikimi et al. (8) described the subcellular distribution of a kinin-forming activity and kininogen in the rat brain. This kinin-forming activity was said to be highest in the cerebral cortex with lower amounts measured in the brainstem and cerebellum. In contrast, kininogen content of the cerebral cortex was described as one-third that of the cerebellum or brain stem. Recently, Shishkova et al. (9) reported the presence of a rat brain kinin-forming activity which was inhibited by aprotinin, but not by SBTI or ovomucoid trypsin inhibitor. Finally, another kininogenase activity in the porcine anterior pituitary gland has been characterized by Powers and Nasjletti (11). This putative enzyme differs from tissue kallikrein in its sensitivity to inhibitors and substrate specificity, as well as the kinin product formed. This body of preceding work suggested the existence of tissue kallikreins and kallikrein-like enzymes within the mammalian brain, which is now supported by the present study.

The cellular localization of this brain kallikrein is a question of great interest, in light of some recent studies of tissue kallikreins and kallikrein-like enzymatic activities. Nolly and Lama (4) have found a kininogenase activity similar to tissue (and not plasma) kallikrein in mesenteric vasculature. If confirmed, this finding must be considered in order to determine whether brain kallikrein is resident in some portion of the central vasculature. The availability of both polyclonal and monoclonal antibodies to tissue kallikreins should allow definitive immunohistochemical identification and radioimmunoassay quantitation of kallikrein concentrations in vasculature, specific brain nuclei, tracts or, perhaps, supporting tissue. Specific localization will then allow further consideration of function in relation to specific proteolysis leading not only to local kinin generation, but perhaps to proteolytic functions that are nondonkin related. These latter kallikrein capabilities (38-40) are of increasing interest since tissue kallikrein is now known to be quite similar to the nerve growth factor γ subunit, the β nerve growth factor endopeptidase, the epidermal growth factor binding protein, and tonin (41, 42). In addition, it is reasonable to suggest that some sort of kallikrein-like enzyme may be involved in pro-opiomelanocortin cleavage, since Lys-Arg and Arg-Set bond cleavage is involved in product generation from this substrate (43, 44) and a serine protease inhibitor (β-butoxycarbonyl-D-Phe-ProArgH) blocks the release of these peptide products (45). It would be of interest to know whether purified tissue kallikreins can release active endocrine peptides from this precursor.

Note Added in Proof—Powers and Nasjletti (46) have described recently a tissue kallikrein-like activity in the rat putative pars intermedia.

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