Existence of a Human Urinary Trypsin Inhibitor (Urinastatin)-Like Substance in the Rat Brain

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ABSTRACT — A human urinary trypsin inhibitor, urinastatin (UT)-like immunoreactive substance with trypsin inhibitory activity, was demonstrated in certain brain regions in rats, especially the cerebral cortex, hippocampus and hypothalamus. Although this UT-like substance in the rat brain displayed an *N*-terminal amino acid sequence similar to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), it did not show any GAPDH activity. These results indicate that the UT-like substance in the rat brain is a protein different from GAPDH and indicates a localized distribution within certain brain regions partly related to learning and memory.

Keywords: Urinary trypsin inhibitor (human), Urinastatin, Brain (rat), Glyceraldehyde-3-phosphate dehydrogenase

Urinastatin (UT) is a human urinary trypsin inhibitor, which is excreted within a range of several mg/day via the urine of healthy humans (1). We have previously found a UT-like immunoreactive substance with trypsin inhibitory activity in the murine urine (2), kidney (3), liver (4) and brain (5). Although this UT-like substance shows a specific distribution within certain brain sites in mice (5), little is known about its localization and biological functions in brain tissues of the most common experimental animal, the rat. Despite the fact that the urine of rats contains substances which inhibit trypsin activity, such a UT-like immunoreactive substance has not been detected in rat urine (2). In the present investigation, we therefore determined if any UT-like substances were present in the rat brain.

MATERIALS AND METHODS

Materials

The UT (a kind gift of Mochida Pharmaceutical Co. Ltd., Tokyo) employed was a compound that migrated as a single band in both SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Sephadex gel chromatography. Standard proteins with known molecular weights (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme) and polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad, U.S.A. and Atto Co., Japan, respectively. The following reagents were used: goat anti-rabbit IgG-horseradish peroxidase (Kirkegaard and Perry Lab. Inc., U.S.A.), Vectastain kit (Vector Lab., U.S.A.), trypsin (Miles Lab., U.S.A.), *t*-butyloxycarbonyl-L-glutaminyl-L-alanyl-L-arginine-4-methyl-coumaryl-7-amide (Boc-Gln-Ala-Arg-MCA) (Peptide Institute Inc., Japan), CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Sweden), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from chicken muscle (Sigma, U.S.A.), and 3-phosphoglycerate kinase (Boehringer Mannheim GmbH, Germany).

UT antibody

Anti-UT sera were raised in rabbits as described previously (2). The complement activity in sera was first neutralized by heating the sera at 56°C for 30 min, and the IgG fraction of the anti-UT sera was then obtained by fractionation with Na_2SO_4 prior to passage through a DEAE-cellulose column. Specificity of the UT antibody was the same as described in the previous report (2). In the ELISA using anti-UT IgG (1.6 μg/ml) and goat peroxidase-labelled anti-rabbit IgG (0.2 μg/ml), there was no cross-reactivity with GAPDH at concentrations as high as 3 μg/ml, whereas the lowest detectable concentration of UT was 0.05 μg/ml.

Tissue extract

Male WKY rats (Charles River, Japan), weighing 250–300 g, were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and perfused through the aorta with physiological saline. The brain was removed, weighed and homogenized by a Potter Elvehjem glass homogenizer with 10 volumes of distilled water on ice. The homogenate was centrifuged at 13,000 × *g* for 10 min at 4°C, and the supernatant was used as the brain extract. Protein concentration was measured by the method of Lowry et al. (6) using bovine serum albumin as a standard.

Affinity chromatography

The brain extract was adjusted with concentrated Tris-HCl and NaCl solution to final concentrations of 4 mM Tris-HCl (pH 8.2) and 0.5 M NaCl. This solution was then applied to a CNBr-activated Sepharose 4B column (1 g, bed volume of 2.4 ml) coupled with rabbit anti-UT IgG (15 mg protein) according to procedures described in the manufacturer's manual. The equilibration buffer was Tris-HCl (4 mM, pH 8.2) in 0.5 M NaCl. Acetic acid solution (4 mM, pH 3.6) containing 0.5 M NaCl was used as the elution buffer. The eluate was dialyzed against distilled water before lyophilization.

Assay of trypsin inhibitory and GAPDH activities

Trypsin inhibitory activity was determined using Boc-Gln-Ala-Arg-MCA as a substrate for the enzyme (3). GAPDH activity was assayed in the coupled reaction with phosphoglycerate kinase (7).

Immunohistochemistry

Rats and mice (male ICR strain, weighing 20–25 g) were anesthetized with pentobarbital sodium (60 and 40 mg/kg, i.p. for rats and mice, respectively), and slowly perfused through the heart with 25 ml heparinized saline (heparin USP 10 units/ml) containing 0.5% $NaNO_2$, followed by 100 ml of 4% folmaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). After perfusion, the brain was removed, fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.2) for 1 hr before washing with 0.1 M sodium phosphate buffer (pH 7.2). The washed organs were then placed overnight in a 30% sucrose solution. The brain was frozen with dry-ice and slices of 30-μm thickness were sectioned using a Histostat (American Optical Co., U.S.A.). The polyclonal rabbit anti-UT serum was used as a primary antiserum, which was diluted in 0.1 M phosphate-buffered saline (pH 7.2) with 0.5% bovine serum albumin to a dilution of 1 : 30,000. The immunoperoxidase procedure was performed according to the manual of the Vectastain ABC kit.

Immunoblot analysis

For SDS-PAGE, a 3% stacking gel, 12.5% separation gel and electrode buffer were prepared according to Laemmli (8). Samples were electrophoresed for 1 hr at 20 mA per slab (9 × 8 cm, 1-mm thickness). Electrophoretic transfer of proteins from the polyacrylamide slab gel to a PVDF membrane was carried out with a Horiz blot (Atto, Japan) using buffer containing 100 mM Tris, 192 mM glycine and 5% methanol at 2 mA/cm^2 for 90 min. For determining the *N*-terminal amino acid sequence, the following discontinuous buffers were used according to the manual of Atto's Horiz blot: 300 mM Tris and 5% methanol; 25 mM Tris and 5% methanol; 25 mM Tris, 40 mM norleucine and 5% methanol. After completing the run, the sheet was cut longitudinally between any two appropriate lanes. Some blots were stained with 0.2% Coomassie brilliant blue in 50% methanol and destained subsequently with 50% methanol. However, other blots were blocked for 12 hr at 4°C with 3% bovine serum albumin in phosphate-buffered saline/Tween (PBS-T, 20 mM phosphate buffer, pH 7.2/0.15 M NaCl/0.05% Tween 20), washed with PBS-T, and allowed to react with anti-UT IgG (1.6 μg/ml in PBS) for 2.5 hr at room temperature. After washing with PBS-T (5 times for 5 min each), the membrane was subjected to immunoreaction with peroxidase-labelled goat anti-rabbit IgG (H + L) (0.2 μg/ml in PBS), prior to washing and follow-up reaction with 3,3'-diaminobenzidine (2.5 mg/10 ml in 50 mM Tris-HCl buffer of pH 7.5 containing 30 μl of 30% hydrogen peroxide) at room temperature.

Determination of N-terminal amino acid sequence

A protein band stained with Coomassie brilliant blue was cut-out from the PVDF membrane with scissors. The *N*-terminal amino acid sequence was then determined with Protein Sequencer 477/120 A (Applied Bio-System, U.S.A.) (9).

Sequence homology

Homology of the *N*-terminal amino aicd sequence was examined by the use of Genetyx-CD (Software Development Co., Japan).

RESULTS

A Coomassie stained gel and the corresponding Western blot are illustrated in (A) and (B) of Fig. 1, respectively. Extracts of the whole brain (lane A-2) contained multiple protein bands, whereas Western blot analysis with anti-UT IgG revealed the presence of a UT-like protein (arrow) in the whole brain. This particular protein migrated to a position that resembled

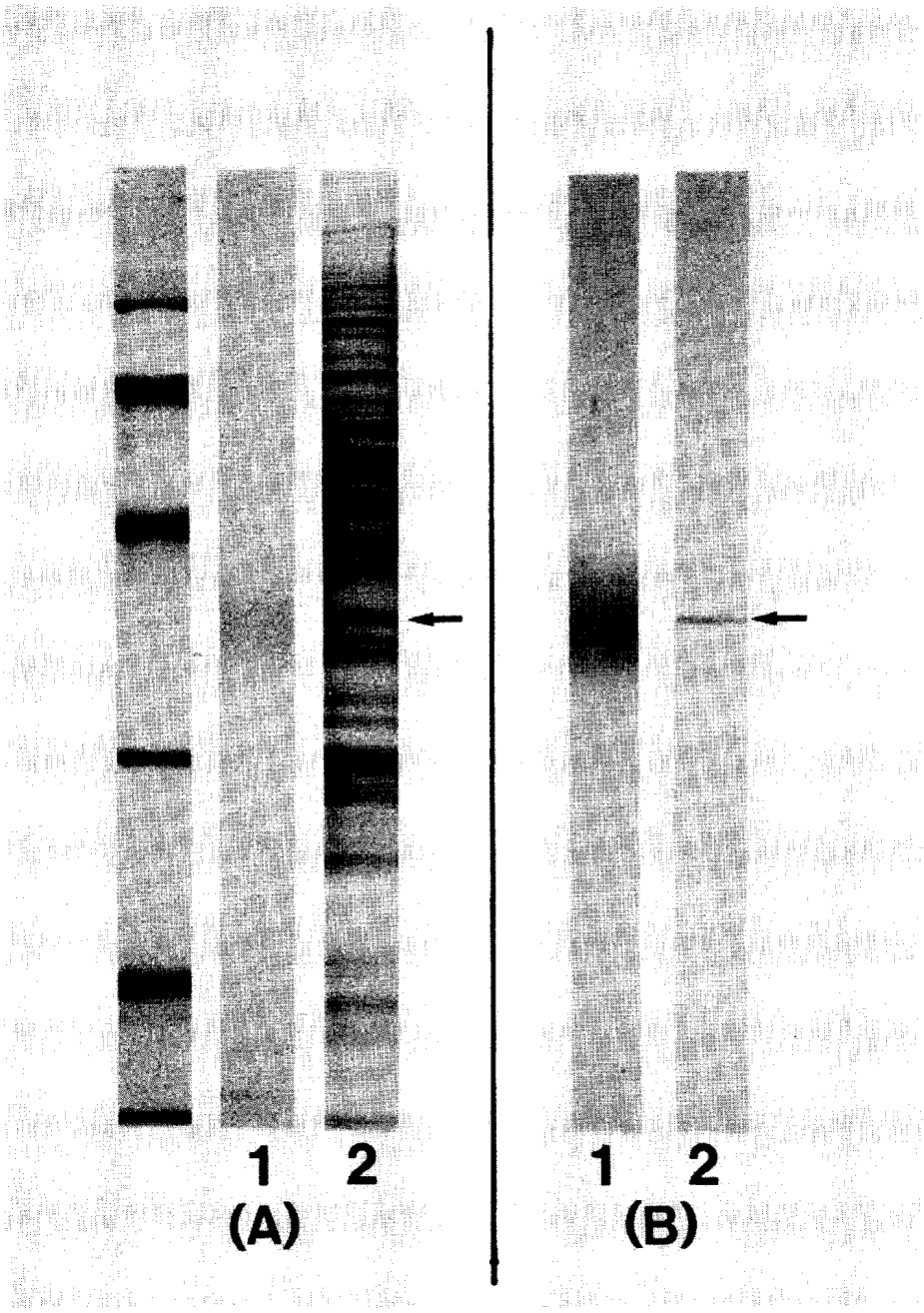

Fig. 1. Detection of urinastatin (UT)-like substance in the rat brain. Samples were heated with 5% 2-mercaptoethanol and 2% SDS at 90°C for 5 min prior to electrophoresis for 1 hr at 20 mA per slab. Coomassie stain (A) and Western blot (B) with anti-UT IgG: lanes 1 and 2 ((A), (B)) are UT (0.6 μg) and rat whole brain extract (17 μg protein), respectively. The unnumbered left lane of (A) shows the marker proteins (bands from top to bottom): rabbit muscle phosphorylase B (Mr = 97,400), bovine serum albumin (Mr = 66,200), ovalbumin (Mr = 45,000), bovine carbonic anhydrase (Mr = 31,000), soybean trypsin inhibitor (Mr = 21,500) and hen egg white lysozyme (Mr = 14,400).

that of UT (compare lane 2 with lane 1 in Fig. 1B).

Immunohistochemically, the rabbit antiserum revealed a regional differentiation of the UT-like immunoreactivity throughout the neuraxis, but the distribution pattern and intensity of the immunoreactivity were similar in mice and rats. Marked stainings were observed in the cerebral cortex, hippocampus and hypothalamus (Fig. 2). In both rats and mice, the pyramidal cells of the cerebral cortex and hippocampus exhibited distinct levels of immunoreactivity (Fig. 3).

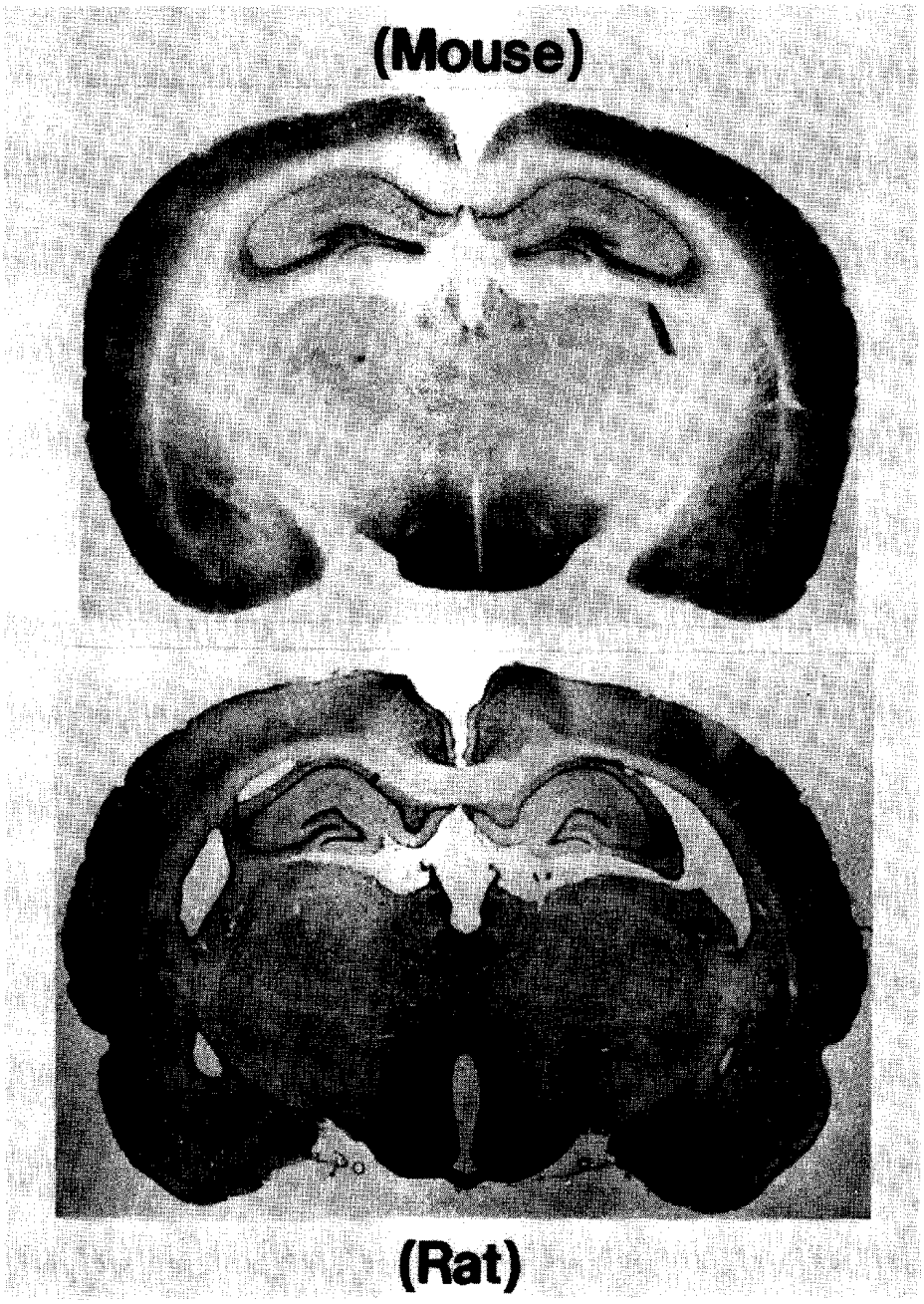

Fig. 2. Immunohistochemical localization of the urinastatin-like substance. Frontal sections of the mouse (upper) and rat (lower) at the level of the posterior third ventricle.

However, brain sections incubatd with preimmunized sera, or alternatively without antisera, were devoid of any immunohistochemical staining.

A Coomassie brilliant blue-stained protein, corresponding well to a protein that reacted with anti-UT IgG, was cut-out from the PVDF membrane (shown with arrow in Fig. 1, lane A-2), since the band was distinctly separated from the other protein bands. The *N*-terminal amino acid sequence of the protein was identified as V-K-V-G-V-N-G-F-G-R-I-G-R-L-; this sequence of 14 amino acids coincided well with those of the GAPDHs of rat muscle and human liver (Fig. 4).

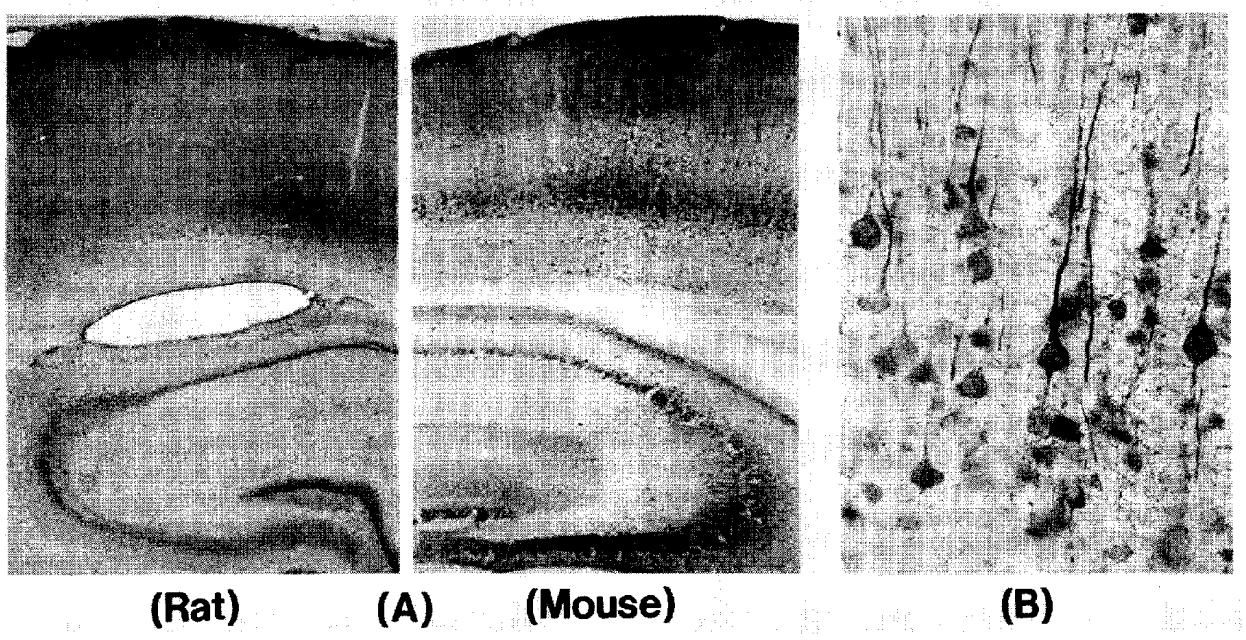

Fig. 3. Immunohistochemical localization of a urinastatin-like substance in the cerebral cortex and hippocampus. Staining pattern in the dorsal hippocampus and overlying cortex (A), and higher magnification of stained apical dendrites of pyramidal cells in the cerebral cortex of rats (B).

Fig. 4. The homology of *N*-terminal amino acid sequences between the anti-urinastatin IgG immunoreactive protein (1) and glyceraldehyde-3-phosphate dehydrogenase (from top to bottom: rat muscle (2), human muscle (3) and human liver (4)). Asterisk represents the same amino acid shown above.

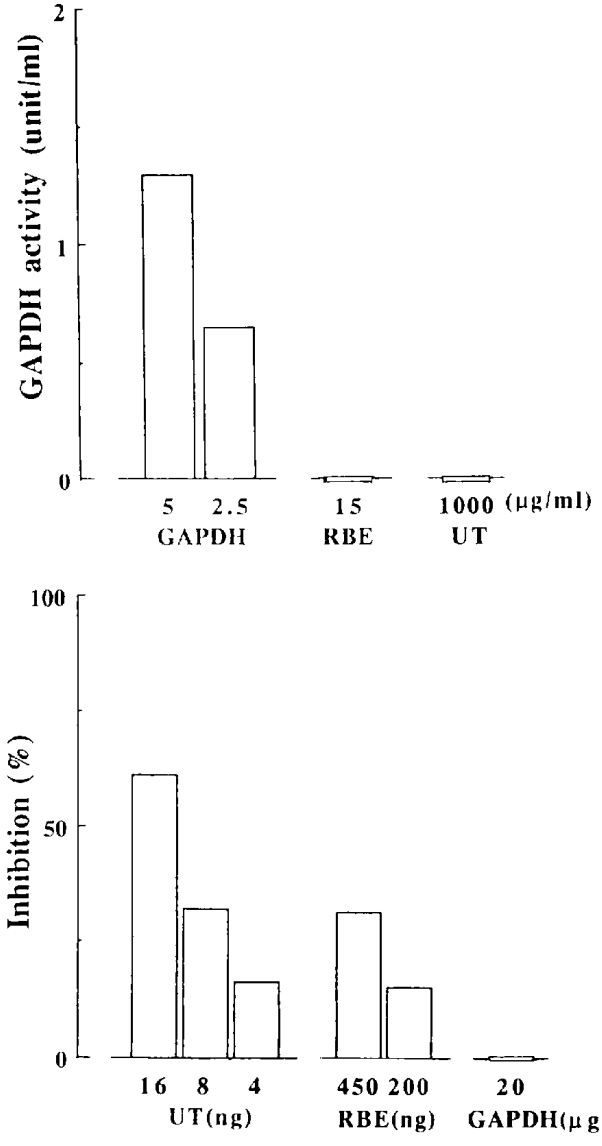

Fig. 5. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity (upper panel) and trypsin inhibitory activity (lower panel). In the upper panel, one unit/ml sample was calculated from the following equation: $(V \cdot \Delta E \div \varepsilon \cdot d \cdot v)/\Delta t$, where V, v, d, ε, ΔE, Δt represent the assay volume (ml), volume of sample used in assay (ml), light path (cm), extinction coefficient ($\varepsilon_{340} = 6.22$ $cm^2/\mu mol$), absorbance change at 340 nm, interval between measurements (min), respectively. In the lower panel, inhibition % was calculated from the difference in trypsin activities with and without sample. Chicken muscle GAPDH (GAPDH), eluate of rat brain extract from anti-UT IgG affinity column (RBE), and urinastatin (UT) were used in the experiment.

Whether or not the anti-UT IgG immunoreactive protein displayed any GAPDH activities, eluates of the whole brain extract from the anti-UT IgG affinity column were examined. Although the standard preparation (2.5 μg of GAPDH) demonstrated a distinct GAPDH activity, the eluate did not show any GAPDH activities even with a sixfold higher amount than that of the GAPDH preparation. By using the same eluate, the tryptic hydrolysis of Boc-Gln-Ala-Arg-MCA was evidently inhibited (Fig. 5). Standard UT (1–1000 μg/ml) and GAPDH (0.1–20 μg) preparations did not elicit any GAPDH and trypsin inhibitory activities, respectively.

DISCUSSION

UT, a glycoprotein with a molecular weight of 40 kDa which migrated as a diffuse band on SDS-PAGE due to the heterogeneity of the carbohydrate moiety (10), has two functional domains; Domain 1 inhibits chymotrypsin and elastase, whereas Domain 2 inhibits trypsin (11, 12). These two domains are highly homologous in their amino acid sequences to the protease inhibitory domain of the amyloid β-protein precursor (13–15). In the present study, the UT-like immunoreactive protein was distributed primarily in the cerebral cortex, hippocampus and hypothalamus, brain regions which are considered to relate partly to memory and learning. In the cerebral cortex and hippocampus, the pyramidal cells were distinctly stained with anti-UT serum. The UT-like substance in urine originates from the precursor protein of UT which is synthesized in the liver (4, 16), whereas the UT-like substance in the brain is probably produced within the neurons (5) and/or astrocytes (17). In the murine brain, rapid and intense UT-like immunoreactivities were observed in the lesioned areas with more intense staining in local neuronal processes (5). Since protease inhibitors derived from neuronal cells function as regulators of neurite elongation and regeneration (18, 19), the secreted UT-like substance may not only display a protective function in limiting focal damage in the specific brain regions due to its protease inhibitory activity, but it may also elicit other effects.

The possibility of the coelution of GAPDH with the UT-like substance from the anti-UT IgG affinity column can be excluded from the findings that anti-UT IgG did not cross-react with GAPDH as described in the Methods section, and a single protein band with a similar apparent molecular weight of about 40 kDa was stained with silver after SDS-PAGE of the eluate (data not shown). The protein, which reacted with anti-UT IgG, displayed a *N*-terminal amino acid sequence similar to that of GAPDH, in which the *N*-terminal portion composes a NAD^+-binding domain (20), besides showing a trypsin inhibitory activity devoid of GAPDH activity. The *N*-terminal amino acid sequence of the UT-like substance differed from that of UT: In UT,

A-V-L-P-Q-E-E-E-G-S-G-G-G-Q- is the *N*-terminal amino acid sequence (21). In both cases where the brain extracts were treated with and without mercaptoethanol before application to SDS-PAGE, the protein showed a similar apparent molecular weight of about 40 kDa. Unlike this UT-like substance, GAPDH manifested different molecular weights under the reduced and non-reduced conditions. This discrepancy may have been attributed to the four identical subunits present in GAPDH (20). Our present results indicated that the UT-like immunoreactive substance was not GAPDH per se. In addition, our findings that a UT-like substance exists in rat brain supports the concept that the rat is a suitable species for research on the biological functions of UT-like substance in the brain. The elucidation of the complete amino acid sequence of UT-like substance in the rat brain is currently under investigation in our laboratories.

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