Identification of tonin in brain and exocrine tissues and in the cell-free translation products encoded by the mRNA of these tissues

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Tissue-specific expression of the esteropeptidase tonin [EC 3.4.99.-] was investigated in rat brain, submandibular gland, pancreas and kidney. Specific polyclonal and monoclonal antibodies to purified rat tonin from the submandibular gland have been developed and characterized and have been purified via a tonin–agarose affinity column. Immunoreactive tonin was measured by a recently developed tonin direct radioimmunoassay using a rabbit tonin antiserum. Resulting tonin levels were found to be $105.27 \pm 2.71 \mu\text{g}/\text{mg}$ (of protein) in submandibular gland, $3.18 \pm 0.32 \text{ ng}/\text{mg}$ in pancreas, $1.35 \pm 0.08 \text{ ng}/\text{mg}$ in kidney and $0.12 \pm 0.01 \text{ ng}/\text{mg}$ in brain (means $\pm$ S.E.M.). Western-blot analysis shows that affinity-purified anti-tonin antibody binds to a 32000- M_r protein from brain and submandibular-gland extracts. The protein, a tonin precursor, was identified from cell-free translation products directed by polyadenylated [Poly(A)⁺] mRNA species in a wheat-germ system. After the translation products were subjected to immunoprecipitation with affinity-purified tonin antibody, SDS/polyacrylamide-gel electrophoresis of these precipitates revealed two precursors of tonin, with M_r values of 30000 and 29000, which are encoded by brain and submandibular-gland mRNA; however, only the 30000- M_r preprotonin was encoded by pancreas and kidney mRNA. Collectively, the data show that tonin exists in brain, submandibular gland, pancreas and kidney, and can be synthesized by the mRNA of these tissues.

INTRODUCTION

Tonin (EC 3.4.99.-) is a recently identified esteropeptidase and a member of a multigene family coding for a subgroup of serine proteinases that are involved in the processing of bioactive peptides. Tonin potentially occupies an important role in cardiovascular function, owing to its ability to cleave angiotensin I to form angiotensin II. More importantly, tonin converts directly the renin substrate angiotensinogen to angiotensin II, a potent vasoconstrictor (Boucher *et al.*, 1974; Schiffrin & Genest, 1983). Despite its similar substrate specificity, tonin is unrelated to the angiotensin I-converting enzyme and is different from renin (Boucher *et al.*, 1977). Other members of this multigene family cleave other biologically active polypeptides: kallikrein cleaves kininogen to release bradykinin or lysylbradykinin (Fiedler, 1979); the γ -subunit of nerve growth factor processes the precursor of nerve growth factor (Thomas *et al.*, 1981); γ -renin cleaves the synthetic renin substrate tetradecapeptide (Poe *et al.*, 1983); and epidermal-growth-factor-binding protein processes the precursor of epidermal growth factor (Frey *et al.*, 1979).

In the rat, the submandibular gland contains the highest levels of tonin as well as the other related esteropeptidases (Boucher *et al.*, 1972; Gutkowska *et al.*, 1982; Shih *et al.*, 1986). Tonin has also been identified immunohistochemically in other rat tissues, such as kidney (St. Ledoux *et al.*, 1982), lung and testes, as well as in plasma (Boucher *et al.*, 1972) and saliva (Garcia *et*

al., 1975), and recently a tonin-like enzyme has been described in human prostate (Watt *et al.*, 1986). Ashley & MacDonald (1985) have also characterized the cDNA for tonin and shown by Northern-blot analysis its expression in submandibular gland and prostate.

We report here the identification of tonin and tonin mRNA in rat brain, submandibular gland, pancreas and kidney by a tonin direct radioimmunoassay, Western blotting and immunoprecipitation of tonin precursor products from translation of poly(A)⁺ mRNA species *in vitro*.

MATERIALS AND METHODS

Enzyme isolation and antiserum production

Rat tonin from submandibular gland was purified as described previously (Demassieux *et al.*, 1976). The homogeneity of rat tonin was determined by SDS/polyacrylamide-gel electrophoresis as described previously (Chao & Chao, 1981). Antiserum against purified rat tonin was raised in rabbits in a way similar to that described for rat urinary kallikrein (Chao & Margolius, 1979) or rat urinary esterase A (Chao, 1983). Rat submandibular-gland tonin (5 mg/ml in 0.1 M-Mops, pH 7.5) was coupled to Affi-Gel 15. A 20 ml portion of rabbit anti-tonin antiserum was diluted to 80 ml with 0.1 M-NaCl/0.01 M-sodium phosphate, pH 7.0, and passed through the tonin–agarose affinity column equilibrated with the same buffer. Adsorbed antibodies were

Abbreviation used: poly(A)⁺, polyadenylated.

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then eluted with 0.1 M-acetic acid. Purified tonin or protein A was labelled with ^{125}I by the lactoperoxidase method (Shimamoto *et al.*, 1980). Kallikrein antibody was affinity-purified as described by Chao *et al.* (1983).

Radioimmunoassay measurements

Tonin was measured in rat brain, submandibular gland, kidney and pancreas by a new direct radioimmunoassay (Shih *et al.*, 1986).

Western-blot analyses

Tonin was immunoprecipitated from brain tissue extracts by using affinity-purified rabbit anti-tonin antibody. The immunoprecipitate from brain or total submandibular-gland extract ($\sim 100 \mu\text{g}$ of protein) and the purified tonin ($\sim 3 \mu\text{g}$) were electrophoresed in a 7.5–15%-(w/v)-linear-gradient polyacrylamide gel containing 0.1% SDS. The proteins were then electrophoretically transferred to nitrocellulose as described by Burnette (1981). Electrophoretic transfer was performed at 15 V for 18 h at 4 °C. The unstained blots were soaked for 1 h at 40 °C in a buffer containing 5% bovine serum albumin, 0.15 M-NaCl and 0.01 M-Tris/HCl, pH 7.4, to saturate unoccupied protein-binding sites. The blot was incubated for 2 h at room temperature with affinity-purified rabbit anti-(rat tonin) antibody (5 $\mu\text{g}/\text{ml}$) in a buffer containing 0.15 M-NaCl, 0.005 M-EDTA, 0.05 M-Tris/HCl, pH 7.4, 3% (w/v) bovine serum albumin and 0.05% Nonidet P40. After incubation of the blot with rabbit anti-(rat tonin) antibody, the blot was washed and incubated with ^{125}I -protein A (10⁶ c.p.m./ml) for 2 h at room temperature. The solution was then aspirated, and the nitrocellulose was rinsed with water and then soaked, with shaking, in 0.15 M-Tris/HCl (pH 7.4)/0.005 M-EDTA/0.15 M-NaCl/0.25% gelatin/0.5% Triton X-100/0.1% SDS at room temperature for 2 h. The blot was affixed to a sheet of filter paper and exposed to Kodak X-Omat AR film at -70 °C, a Cronex Lightning Plus intensifying screen being used, for 6–24 h.

Preparation and immunoprecipitation of translation products

Total RNA from brain was prepared as described by Chao *et al.* (1983), whereas RNA from submandibular gland, pancreas and kidney were purified by the guanidinium thiocyanate extraction procedure of Chirgwin *et al.* (1979). Poly(A)⁺ RNA was isolated by two cycles of chromatography on an oligo(dT)-cellulose column. Poly(A)⁺ mRNA was translated in a wheat-germ system (Chao *et al.*, 1983) and immunoprecipitated with affinity-purified rabbit anti-tonin antibody (Woodley *et al.*, 1985). The precipitates were analysed on polyacrylamide gels containing 0.1% SDS and made visible by fluorography.

RESULTS

Tonin levels in tissue extracts

Fig. 1 shows typical log-logit transformations of the radioimmunoassay standard curve of purified rat tonin and of curves of serial dilutions of rat tissue extracts from submandibular gland, kidney and pancreas. Linear curves parallel with the standard curve were obtained, indicating the immunological identity of these tissue enzymes and tonin. Further, tonin was measured

Fig. 1. Log-logit transformation of typical radioimmunoassay standard curves of rat tonin (●), submandibular gland (▲), kidney (△), and pancreas (■)

B/B_0 is the ratio of bound radioactivity in the presence of unlabelled tonin to that in its absence, expressed as a percentage, using a rabbit anti-tonin antiserum at a final dilution of 1:160 000.

Table 1. Rat tissue tonin distribution

Tonin levels were measured by a tonin direct radioimmunoassay using affinity-purified rabbit anti-tonin antibodies. Values shown are means $\pm$ S.E.M. for three determinations in each case.

Tissue	Tonin (amount/ mg of protein)
Salivary	105.27 $\pm$ 2.71 μg
Pancreas	3.18 $\pm$ 0.32 ng
Kidney	1.35 $\pm$ 0.08 ng
Brain	0.12 $\pm$ 0.01 ng

quantitatively by the radioimmunoassay in each of these tissues. Table 1 shows that the tonin level in submandibular gland ('salivary') is five orders of magnitude greater than that in pancreas and kidney, whereas it is six orders of magnitude greater than that in the brain (0.10–0.14 ng/mg).

Western-blot analyses

To demonstrate the presence of tonin, submandibular-gland extract and the immunoprecipitate from brain extract, along with purified tonin, were electrophoresed in a polyacrylamide slab gel under reducing conditions and transferred to nitrocellulose. After incubation with rabbit anti-tonin antibody and binding to ^{125}I -protein A, tonin from each of the tissues was displayed visually by autoradiography (Fig. 2). Similar results were obtained when ^{125}I -labelled monoclonal anti-tonin antibodies were used to identify tonin in these tissues as described by Woodley *et al.* (1985) (results not shown). Western-blot analysis shows that tonin in brain and in submandibular gland is immunologically indistinguishable, with an M_r of 32 000 (Fig. 2).

Fig. 2. Western-blot analysis of rat brain and submandibular-gland tonin

Fresh tissues were homogenized and clarified by centrifugation (see the Materials and methods section). The submandibular-gland extract and brain immunoprecipitate were separated in a linear-gradient (7.5–17.5%, w/v) polyacrylamide gel containing 0.1% SDS and Western-blotted with affinity-purified rabbit anti-tonin antibody and ^{125}I -protein A. The binding was demonstrated by autoradiography. The gel contains purified tonin (lane a), brain-extract immunoprecipitate (lane b) and submandibular-gland homogenate (lane c).

Cell-free translation of rat brain mRNA

Poly(A)⁺ mRNA species from rat brain, submandibular gland, pancreas and kidney were translated in a wheat-germ system. Preprotonin was immunoprecipitated with affinity-purified rabbit anti-tonin antibody from the total translation products of each of these tissues and was electrophoresed on an SDS/polyacrylamide gel (Fig. 3). The immunoprecipitates reveal a 30 kDa primary translation product (lane d) from each of the tissues, whose migration is similar to that of ^{125}I -tonin (submandibular gland, lane e). A second immunoprecipitate band was identified in submandibular gland and brain, with an M_r of ~ 29000 (lane d). In brain (lane e) the 37 kDa peptide is the immunoprecipitate of affinity-purified kallikrein antibody and represents preprokallikrein, a closely related enzyme that cross-reacts immunologically with tonin. Therefore the 37 kDa band in submandibular gland (lane d) and in brain (lane d) likely represents tissue kallikrein.

DISCUSSION

The present study demonstrates the expression of tonin in rat brain, submandibular gland, pancreas and kidney. Further, the enzyme in these tissues is generally indistinguishable with respect to size, charge and immunological characteristics.

Immunological identity and levels of tonin among rat submandibular gland, pancreas, kidney and brain were shown by a tonin radioimmunoassay. Parallel linear

curves of typical log-logit transformation from the standard curve of purified rat tonin and from the curves of serial dilutions of these tissue extracts indicate immunological identity. The levels of tonin, however, varied widely, as shown by a range of six orders of magnitude from a high level in submandibular gland to a low level in brain (Table 1). The high level of tonin in rat submandibular gland is characteristic of this enzyme as well as of other related members of a large family of arginyl-esteropeptidases that include tissue kallikrein (Gerald *et al.*, 1986), γ -subunit of nerve growth factor, epidermal-growth-factor-binding protein, β -nerve-growth-factor endopeptidase (Bothwell *et al.*, 1979) and arginylesterase A (Chao, 1983). The reason for this high level of expression among these family members in submandibular gland is unknown. It is also not known whether the extremely low level of tonin in brain is due to a generalized distribution or due to a regional or cellular distribution which would cause a dilution of tonin when the extract is prepared from whole brain.

Western-blot analysis using affinity-purified polyclonal antibody to rat tonin identifies immunoreactive tonin in brain and submandibular gland. The enzymes were found to be similar, with respect to mobility, to purified tonin, with an M_r of 32000. By using monoclonal antibody to tonin, a 32 kDa protein similar to purified tonin was identified in pancreatic extract by immunoblotting (results not shown).

Tonin precursors were immunoprecipitated and identified from a cell-free translation system. The preprotonin translation products of pancreas, kidney, submandibular gland and brain appear to be similar in size, with an M_r of ~ 30000 . The presence of a second band, in both submandibular gland and brain, with an M_r of 29000 may represent the translation product of 'S3 mRNA' described by Ashley & MacDonald (1985). This S3 mRNA (cDNA), which was isolated from rat submandibular gland, encodes an enzyme closely related to tonin and which was shown to be expressed in prostate as well as in submandibular gland. However, whether this 29 kDa protein is the precursor of a tonin-related peptide or a precursor for the active 32 kDa tonin remains to be determined.

Tonin likely functions within the organ in which it is synthesized, and its presence reflects the need for peptide maturation within a specific cell type or tissue. The expression of tonin in submandibular gland and kidney suggests another source of angiotensin II through the direct cleavage of angiotensinogen or angiotensin I. The co-existence of tonin and kallikrein in the submandibular gland and kidney may suggest a role for a tonin-angiotensin II system in opposing the effects of the kallikrein-kinin system on blood flow and electrolyte transport (Zacharatos *et al.*, 1983). The present finding of tonin expression in the brain suggests several possible functional roles. Tonin has been shown to hydrolyse peptide hormones such as β -lipotropin, adrenocorticotrophic hormone (Seidah *et al.*, 1979), substance P (Chrétien *et al.*, 1980) and kininogen (Ikeda & Arakawa, 1984), and therefore it may be involved in their maturation or metabolism. Further, the expression of tonin in the brain presents the possibility of a new direct pathway of angiotensin II production. The presence of isorenin (Ganten *et al.*, 1975), angiotensinogen, angiotensin I-converting enzyme (Fischer-Ferraro *et al.*, 1971) and angiotensin II (Changaris *et al.*, 1978; Phillips

Fig. 3. Electrophoretic analysis of tonin-immunoreactive peptides from rat submandibular-gland (a), brain (b), pancreas (c) and kidney (d) mRNA-directed translation products

These mRNA species were translated in a wheat-germ system with [35 S]methionine as the radiolabel. Labelled proteins and immunoprecipitates were separated on a linear-gradient (7.5–17.5%, w/v) polyacrylamide gel and revealed by fluorography: lane a, total translation products; lane b, immunoprecipitation products with affinity-purified rabbit anti-tonin antibodies, with no exogenously added mRNA; lane c, total translation product from added poly(A) $^{+}$ mRNA; lane d, immunoprecipitates of translation products with affinity-purified rabbit anti-tonin antibody. In submandibular gland, lane e contains 125 I-tonin; in brain, lane e contains the immunoprecipitate of translation products with affinity-purified sheep anti-kallikrein antibodies. M_r markers were: phosphorylase b, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; soybean trypsin inhibitor, 20100; and α -lactalbumin, 14400.

et al., 1980) in the brain has been reported previously, and it has been assumed that angiotensin II formation in the brain followed the classical pathway. However, the possibility now exists for an alternative pathway of blood-pressure regulation and water balance in the brain through the tonin–angiotensin II system.

The functions suggested for tonin in brain as well as in kidney, submandibular gland and pancreas seem to be among likely functions, but are yet to be elucidated.

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