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Solid-Phase Radioimmunoassay of Tonin in Extracts of Submandibular Glands of Rats Treated Chronically with Isoproterenol (41413)

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Abstract. Tonin, a proteolytic enzyme isolated from rat submandibular gland, can generate angiotensin II directly from angiotensin I, from angiotensin I-tetradecapeptide, and from angiotensinogen. A sensitive and specific solid-phase radioimmunoassay for measurement of tonin concentration and a method for measurement of tonin activity have been developed. Excellent correlation (coefficient $r = 0.95$) was found between the two methods. Tonin concentration and activity were studied in submandibular glands of rats chronically treated with isoproterenol. Almost complete depletion of tonin in rat submandibular gland was observed after 33 days of treatment. This observation was confirmed by polyacrylamide gel electrophoresis of homogenates of rat submandibular gland and immunocytochemistry. The immunocytochemical study revealed the presence of tonin in the cells of the granular convoluted tubules.

Tonin, a proteolytic enzyme of the serine family (1), capable of generating angiotensin II from angiotensinogen, from angiotensin-tetradecapeptide (renin synthetic substrate) and from angiotensin I, has been found in a high concentration in rat submandibular gland (SMG) (2, 3). Tonin has been purified by classical methods (4) and, recently, by affinity chromatography (5).

A radioimmunological method for the measurement of tonin concentration has been developed (6). The method is based on the use of goat anti-rabbit gamma globulin in the separation of free and bound tonin.

In this paper we describe a more sensitive and simpler solid-phase radioimmunoassay of tonin as well as a method of measuring tonin enzymatic activity in homogenates of submandibular glands of rats chronically treated with isoproterenol (IPR). In addition, tonin has been localized immunocytochemically in rat submandibular glands.

Materials. Angiotensin I was purchased from Beckman Inc.; crystalline bovine serum albumin (fraction V) was from Miles Laboratories Inc.

DL-Isoproterenol–HCl (1-[3′,4′-dihydroxyphenyl]2-isopropylaminoethanol–HCl) (No. 1-5627) was purchased from Sigma Chemical Company.

$Na^{125}I$ (specific activity 13–17 Ci/g I) was obtained from New England Nuclear, Boston, Massachusetts.

Tonin. Rat tonin was purified, as described previously, by affinity chromatography (5) from rat submandibular glands. The preparation was found to be homogeneous by commonly used criteria such as polyacrylamide gel electrophoresis, isoelectrofocusing, and gel filtration. This preparation was used for labeling and standard in the radioimmunoassays, without any repurification, after 4–5 months of storage at 4°.

Antisera to tonin were prepared in rabbits by a slight modification of the method used previously (6). Suitable antisera with high titer were obtained after 12 weeks of immunization with two booster injections of 20 μg of tonin. The undiluted antiserum was stored at −80° without preservative and was used without any previous treatment or purification.

Methods. *Solid-phase radioimmunoassay.* The purified tonin was labeled with ^{125}I by the chloramine-T method, as described previously (6). ^{125}I was added at a concentration of 400 μCi/5 μg of tonin. The un-

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bound iodine was separated from protein on Sephadex G-15.

Preparation of coated tubes. The coated tubes were prepared as follows (7). To each polystyrene LKB tube, 0.9 ml of antiserum diluted 1:80,000 in 0.05 *M* sodium carbonate buffer, pH 9.5, was added. The coating process was allowed to proceed for a minimum of 4 hr or overnight at 4°. The antiserum was removed by suction and the tubes were rinsed once with 2 ml of 0.1 *M* phosphate buffer, pH 7.4. To prevent nonspecific adsorption of tonin, 2 ml of 0.1% BSA in 0.01 *M* phosphate buffer, pH 7.4, was added and the tubes were incubated for 1–2 hr at 4°. The tubes were then rinsed twice with 2 ml of 0.01 *M* phosphate buffer, pH 7.4, and used immediately or stored with rinsing buffer at 4°.

Radioimmunoassay procedure. All reagents were diluted in 0.1 *M* phosphate buffer, pH 7.4, 0.25% (w/v) bovine serum albumin, and 0.02% sodium azide. A standard curve was established with tubes (in duplicate) containing from 0 to 2.5 ng of tonin. After careful removal of rinsing buffer, the reactants were added to antibody-coated tubes in the following sequence: 100 μl of standard or sample, 100 μl of radioactive tonin (~6000 cpm) made up to 900 μl with the radioimmunoassay buffer. The tubes were mixed in a Vortex mixer and incubated for 48 hr at 4°. The solution was then carefully removed by suction; the tubes were rinsed twice with 2 ml of 0.01 *M* phosphate buffer, pH 7.4.

Radioactivity bound to antibody was quantitated in a LKB gamma counter (Model 1270 Rackgamma). All values were corrected for nonspecific counts, by substracting the counts measured in BSA-coated tubes in the absence of antitonin.

The general analysis of data was performed by linearization, the standard dose–response curve being the logit transformation of B/B_0 versus logarithm of tonin concentration (B = bound counts for an experimental point of the standard curve, B_0 = the quantity of bound radioactivity for zero dose).

Generally, homogenates of SMG were diluted 1:10,000 with standard RIA buffer and assayed in duplicate at different levels, e.g., 50 and 100 μl. Different dilutions of homogenates of SMG were tested against the standard curves for parallelism.

Measurement of tonin activity. Tonin activity was measured as the rate of generation of angiotensin II from angiotensin I at 37°. Different dilutions of homogenates of submandibular glands, in 100-μl volumes, were incubated with 100 μl of angiotensin I solution (1 μg/ml) in a total volume of 1 ml of 0.1 *M* phosphate buffer, pH 6.8, containing 8 m*M* 8-OH quinoline, 10^{-3} *M* EDTA, 10^{-3} *M* DFP, and 10^{-3} *M* dipyridyl. Aliquots (100 μl) were taken out after 30 and 60 min of incubation, respectively, and were assayed for angiotensin II by radioimmunoassay. Our anti-angiotensin II antibody has 0.4% cross-reactivity with angiotensin I. The submandibular gland homogenates and angiotensin I solutions were assayed as controls and the results were corrected accordingly.

Isoproterenol stimulation. Male Fisher rats weighing 250–300 g were used. The rats had free access to a standard commercial diet and were given tap water *ad lib.* Ten control rats received twice daily (at 8 AM and 4 PM) an intraperitoneal injection of 0.9% NaCl in water. Rats were placed on a regimen of two daily intraperitoneal injections of 25 mg of isoproterenol (IPR) in 0.9% NaCl for periods of 6, 21, or 33 days. In the two last groups the animals were not injected during weekends. The solutions of IPR were freshly prepared before each injection.

After the last injection, the animals were anesthetized with ether and submandibular glands were quickly excised, weighed, and kept on ice.

Preparation of homogenates. The 5% homogenates of submandibular glands were prepared in 0.1 *M* Tris–HCl buffer, pH 7.4, using a Polytron homogenizer. Homogenates were centrifuged for 30 min at 105,000*g*. Aliquots of the supernatants were used for the measurement of protein, tonin concentration, and activity.

Measurement of protein. Protein concentration was determined by the method of Bradford (8) using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis was performed according to a modification of the method of Davis (9). The electrophoretic column measured 0.5 × 9.0 cm. Concentrations of 10 and 1.5% polyacrylamide were used for the running gel and concentration gel, respectively. The supernatants (ca. 150–220 μg protein) in 0.1 M phosphate buffer, pH 7.4, were applied to each gel and electrophoresis was run at 3 mA per column for 120 min at pH 8.3. The gels were stained with Coomassie Blue R-250.

Immunocytochemistry. The submandibular glands from control and isoproterenol-treated rats were fixed for 24 hr in Bouin's fluid, embedded in paraffin, and cut at 5 μm. The sections were deparaffinized and stained according to the unlabeled antibody technique of Sternberger (10). They were exposed to the primary antiserum (6) diluted 1:100 for 48 hr. To test the specificity of the immunocytochemical reaction, four types of controls were used:

1. Exposure to the diaminobenzidine–H_2O_2 step without any incubation with antiserum.
2. One of the components of the stain was left out.
3. Normal rabbit serum was substituted for the specific antiserum.
4. The specific antiserum was preadsorbed with pure submandibular gland tonin (0.25 ml, 1:100 antiserum, 5 ng, 5 μg of tonin) at 4° for 17 hr with constant agitation.

Results. *Solid-phase RIA.* To search for the optimum concentration of antiserum for coating polystyrene tubes, a series of different dilutions ranging from 1:10 to 1:100,000 was used. Figure 1 shows the percentage of binding at different concentrations of antiserum and the effects of concentrations of antiserum on competition between labeled and unlabeled tonin.

A dilution of 1:80,000 for this particular antiserum was required to achieve 40–50% binding and a sensitive standard curve.

Attachment of antitonin diluted 1:80,000 (900 μl per tube) to the plastic tubes was time dependent. The maximum binding was obtained after 3–4 hr at 4° and was not changed on longer incubation. The pH of

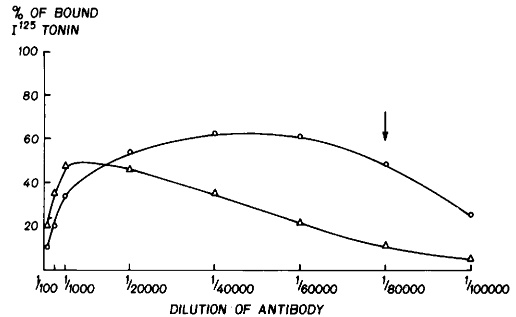

FIG. 1. Variation in capacity of antibody-coated tubes to bind radioactive tonin (^{125}I-tonin). ○, Percentage of ^{125}I-tonin bound by different dilutions of antibody; △, percentage of ^{125}I-tonin bound to antibody in the presence of 2500 pg of unlabeled enzyme per tube, expressed as B/B_0.

the buffer used for dilution of the antibody had no significant effect between pH 6.0 and pH 10.0.

The relationship between logit B/B_0 and log of tonin concentration was linear. The lowest detectable amount of tonin which differs from zero by two standard deviations was 39 pg.

The binding properties of antitonin were tested at different pHs. The standard curves obtained at pH 6.0, 7.0, and 8.0 were superposable.

The specificity of this antiserum was determined as described previously (6). No cross-reactivity of antitonin was observed with rat and pig renin, renin substrate, trypsin, urinary kallikrein (11), chymotrypsin, subtilisin, angiotensin I, and angiotensin II.

Accuracy of the assay was determined by adding different quantities of tonin (5 and 20 ng) to homogenates of rat submandibular glands. The samples were assayed and recoveries for each addition were calculated. There were no significant differences between mean recoveries (82–95%) for each quantity of added tonin.

The intraassay coefficient of variation was 7.8% and the interassay coefficient of variation (15 samples) was 10.2%.

A linear correlation was observed between values of tonin concentration obtained by direct radioimmunoassay and values of tonin activity determined by enzymatic assay in the same samples. The correlation coefficient was 0.95.

Effect of isoproterenol stimulation. The weight of submandibular glands (Table I) doubled by the sixth day of the administration of isoproterenol. With prolonged treatment, the average weight of glands increased about three times, without marked changes in protein concentration as expressed in milligrams of protein per gram of tissue. The content of tonin in submandibular glands decreased significantly after 6 days of treatment. After 33 days of treatment with IPR, the submandibular glands were almost completely depleted of tonin. Depletion of the gland was also confirmed by electrophoresis of homogenates of submandibular glands (Fig. 2). The band of protein corresponding to tonin was present only in control rats.

Immunocytochemistry. In control rats, the staining was strictly limited to the cells of the granular convoluted tubules. Although the intensity of staining varied slightly from cell to cell, it was generally very marked and covered the whole cytoplasm except the nucleus (Fig. 3). In animals treated for 6 days the same localization of staining was observed but it was much weaker, being in most cases restricted to a small portion of the cytoplasm (Fig. 4). Staining was not present after exposure to the diaminobenzidine–H_2O_2 step alone; it was likewise not present when one of the components of the stain was left out, or when normal rabbit serum was used, or when the antiserum had been adsorbed with pure tonin (5 μg).

Discussion. In the present paper we describe a solid-phase radioimmunoassay of tonin, and a highly sensitive method for measurement of tonin activity; the former method is based on competition between labeled and unlabeled tonin for antibody coated on plastic tubes. The present solid-phase method is quick and simple, the results being obtained in 48 hr. The sensitivity of solid-phase RIA is greater than the previously described double-antibody method (6), since with the former technique the lowest detectable quantity of tonin is 39 pg in comparison with 156 pg with the double-antibody method. The recovery of tonin added to homogenates of SMG was 82–

TABLE I. THE EFFECT OF CHRONIC ISOPROTERENOL TREATMENT ON TONIN CONCENTRATION AND ACTIVITY IN RAT SUBMANDIBULAR GLANDS

	IPR treatment 6 days		IPR treatment 21 days		IPR treatment 33 days	
	Controls	Experimental	Controls	Experimental	Controls	Experimental
Body weight						
Beginning of experiment (g)	272 ± 23[a]	279 ± 21	298 ± 18	296 ± 30	—	—
End of experiment (g)	266 ± 20	258 ± 24	293 ± 37	308 ± 25	—	—
Weight of SMG (g)	0.37 ± 0.08	0.72 ± 0.05	0.41 ± 0.04	1.20 ± 0.2*	0.53 ± 0.09	1.49 ± 0.23*
Protein concentration in SMG (mg/g tissue)	44.0 ± 26	39.7 ± 4.8	43.2 ± 4.4	47.9 ± 4.5	40.4 ± 5.4	42.9 ± 4.4
Tonin concentration (μg/g tissue)	6900 ± 1290	934 ± 712*	5360 ± 2280	52.7 ± 53*	4370 ± 1890	0.99 ± 0.07*
Tonin activity (μg AII/g tissue/30 min)	2820 ± 1170	390 ± 236*	3500 ± 853	24.7 ± 26.3*	2480 ± 1290	0.44 ± 0.07*
Number of animals	10	12	10	15	14	18

[a] All values are mean ± SD.
* $P < 0.005$.

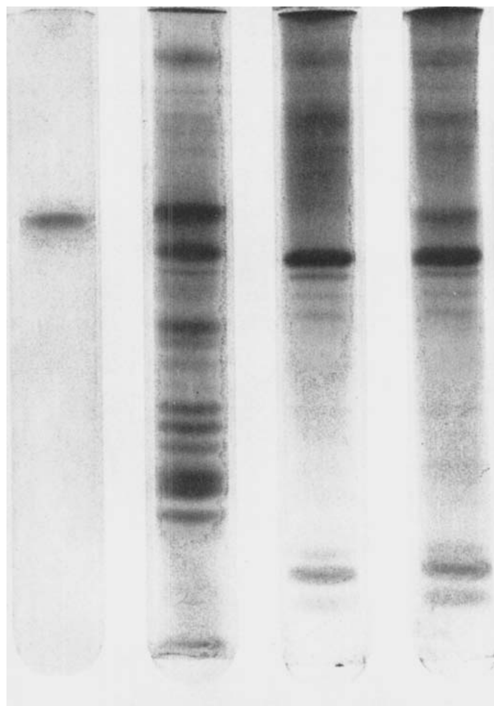

FIG. 2. Polyacrylamide gel electrophoresis analysis of supernatants of submandibular glands. Column: (1) Purified tonin (20 μg); (2) supernatant of SMG of control animals (~200 μg protein); (3) supernatant of SMG of animals treated 33 days with IPR (~200 μg protein); (4) supernatant of SMG of animals treated 33 days with IPR plus added tonin (~200 μg protein and 20 μg tonin).

95%, while the assay coefficient of variation was 7.8–10.2%.

The antiserum showed good specificity in cross-reactivity studies. An additional indirect proof of specificity is the very good correlation obtained when tonin concentration values were compared with the values of tonin activity measured by enzymatic assay.

The submandibular glands of mouse and rat and also of several other species contain a bewildering array of physiologically active peptides, many of which are still without known function (12). Most of these polypeptides are contained in the duct cells, particularly in granular convoluted tubules, which constitute a modified segment of the striated ducts (12). The present immunocytochemical results indicate that tonin is also localized in the granular convoluted tubular cells.

The β-adrenergic agent isoproterenol induces the synthesis of DNA and division of the acinar cells as well as secretion of various proteins from submandibular glands (13–15). Chronic stimulation with the drug results in stimulation of protein synthesis and hyperplasia and hypertrophy of the acinar cells with concomitant relative decrease in the number of duct cells (16–19). This increased protein synthesis reflects the addition of new cells and not excess protein accumulation, since we did not find a greater protein level in chronically treated rats than in control animals at any time after administration of IPR. The dose of IPR we have selected produced a slight decrease in body weight and a significant enlargement of submandibular glands as early as 6 days after the beginning of treatment. Our results are entirely in agreement with those of other workers (14, 16, 20, 21). Isoproterenol also induces a striking decrease in tonin concentration in the gland, confirmed by immunocytochemistry and electrophoresis. This is in agreement with the marked increase of tonin in rat saliva following administration of isoproterenol (22). The flow of saliva is also increased by IPR (16). Although protein synthesis is not as active in granular cells as in acinar cells, it does exist and is higher after acute degranulation by pilocarpine (23). Our results suggest that under chronic stimulation by IPR, synthesis does not keep pace with secretion and storage is minimal. This is not always the case, however, and a different picture emerges from studies with kallikrein, which is also stored and probably produced in granular duct cells: after chronic treatment of rats with isoproterenol, kallikrein concentration in submandibular gland is only slightly lower (24). The secretion of kallikrein in saliva induced by isoproterenol is, however, much less pronounced than that of tonin (24). At any rate, our results indicate that tonin synthesis is independent of acinar cell activity and this enzyme, as demonstrated by immunocytochemistry, may be regarded as an exclusively ductal product.

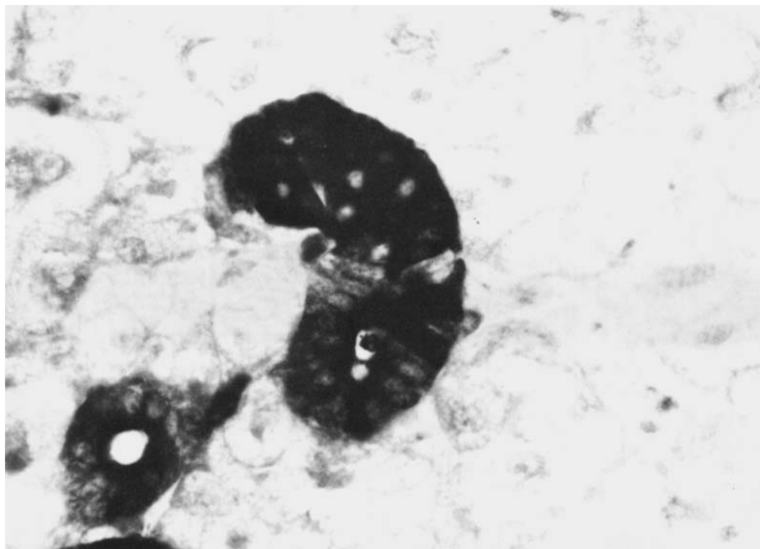

FIG. 3. Section of submandibular gland of control rat stained according to the unlabeled antibody technique of Sternberger. Note intense staining of the whole cytoplasm of granular convoluted tubular cells ($\times$400).

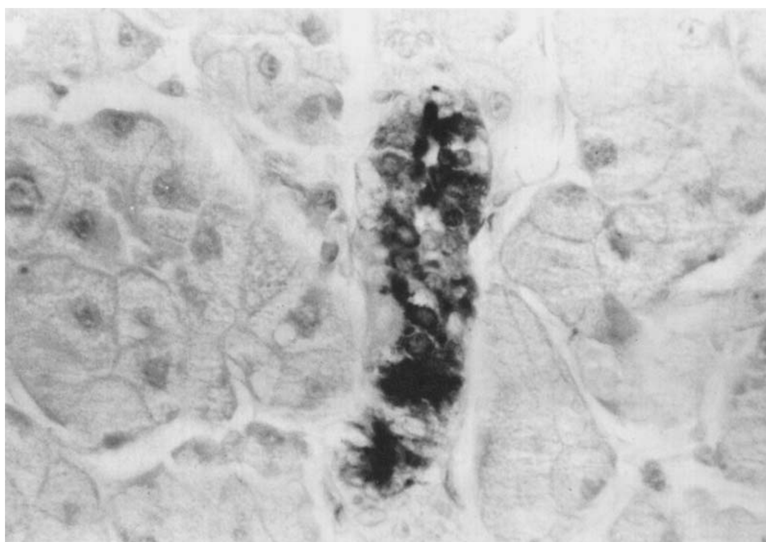

FIG. 4. Section of submandibular gland of rat treated with isoproterenol for 6 days, stained as above. The staining in granular convoluted cells is spotty ($\times$400).

We can only speculate at this stage on the function of tonin in submandibular glands. It may be involved in the local formation of angiotensin II (25) or may have, as do so many of the "biologically active polypeptides" found in submandibular glands, more important systemic effects on blood pressure and electrolyte regulation through the formation of plasma angiotensin II.

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