In Vitro Secretion of Immunoreactive Tonin from Dispersed Rat Submandibular Gland Cells

YUKIO HIRATA, MASAHIRO TOMITA, TAKUO FUJITA, AND MASAO IKEDA

SUMMARY Dispersed cells from the submandibular gland of the male rat were prepared by collagenase treatment to study the mechanism by which immunoreactive tonin is secreted in vitro. Norepinephrine, epinephrine, and phenylephrine stimulated tonin release, an effect that was inhibited by phentolamine but not by propranolol, whereas isoproterenol, carbacol, histamine, and serotonin did not stimulate tonin release. The stimulatory effect elicited by α-adrenergic agonists was inhibited by both removal of Ca^{2+} from the medium and addition of diltiazem and nifedipine, both selective calcium channel blockers. The divalent cation ionophore A23187 stimulated tonin release in the presence of Ca^{2+}, but not in the presence of Mg^{2+}. Dibutyryl cyclic adenosine 3',5'-monophosphate, methylisobutylxanthine, angiotensin II, and vasoactive intestinal peptide had no effect on tonin release. The apparent molecular size of immunoreactive tonin released into the medium under basal and norepinephrine-stimulated conditions was similar to that of standard tonin by gel exclusion chromatography. These data suggest that the in vitro secretion of immunoreactive tonin from rat submandibular gland is initiated by activation of α-adrenergic receptors and apparently involves a mechanism dependent not on cyclic adenosine 3',5'-monophosphate, but on the influx of extracellular Ca^{2+}. (Hypertension 8: 883-889, 1986)

KEY WORDS • tonin • in vitro secretion • rat submandibular gland • dispersed cells

RAT submandibular gland (SMG) contains tonin, an enzyme of the serine protease family that acts directly on angiotensin (ANG) I, angiotensinogen (renin substrate), or a synthetic tetradecapeptide renin substrate to yield ANG II, a potent vasoconstrictive hormone. Synthesis of SMG tonin appears to be under androgenic control, \(^{1,2}\) since levels of SMG tonin in the normal male rat exceed those in normal female rats, levels in castrated male rats fall to those in normal female rats, and levels in testosterone-treated female rats increase to levels in normal male rats. On the other hand, isoproterenol, a β-adrenergic agonist, has been reported to stimulate release of tonin into saliva, an effect that may be mediated by intracellular cyclic adenosine 3',5'-monophosphate (cyclic AMP). \(^{3,4}\) A more recent study has shown that tonin activity is secreted into the venous effluent from the perfused rat SMG after β-adrenergic stimulation. \(^{5}\)

To study the detailed mechanism by which tonin is secreted from rat SMG, we prepared SMG cells dispersed by collagenase from male rats and investigated the effects of a variety of agents on the release of immunoreactive tonin in vitro and the possible intracellular process responsible for its release.

Materials and Methods

Incubation Procedure

Adult (8-10 weeks old) male Wistar rats weighing 250 to 350 g were used (Kitayama LABES, Kyoto, Japan). The SMGs were removed from two rats between 0900 and 1000 under sodium pentobarbital anesthesia. The glands were rinsed several times with ice-cold Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.2% glucose and 0.1% bovine serum albumin, and minced with curved scissors. The minced tissue was suspended in 50 ml of the buffer containing 0.2% collagenase (Type IV; Worthington Biochemical, Freehold, NJ, USA) and slowly stirred for 60 minutes at 37°C in an atmosphere of 95% O\(_2\), 5% CO\(_2\) in a 250-ml suspension culture flask (Celstir; Wea-
ton, Millville, NJ, USA). The cell preparation was centrifuged at 100 g for 5 minutes at 4°C, the supernatant was aspirated, and the cell pellet was washed three times with 50 ml of ice-cold Krebs-Ringer bicarbonate buffer. The cells then were resuspended in the buffer, and the cell count (as determined by a hemocytometer) was adjusted to 1 to 5 X 10⁶ cells/ml. Aliquots (0.9 ml) of the cell suspension then were pipetted into 12 x 75-mm polystyrene tubes, and 0.1 ml of test samples dissolved in Krebs-Ringer bicarbonate buffer or of buffer alone was added to five incubation tubes.

The samples were incubated at 37°C for 60 minutes in an atmosphere of 95% O₂, 5% CO₂. The suspended cells then were centrifuged, and the supernatant was removed for analysis. Cell viability, assessed by trypan blue exclusion was 90 to 95% before and after incubation.

In some experiments, Ca²⁺, Mg²⁺-free buffer was used to study the effect of divalent cations on the release of tonin. The amounts of immunoreactive tonin released into the medium were determined by specific radioimmunoassay (RIA).

Radioimmunoassay and Activity of Tonin

The tonin antibody used in the present study was raised in rabbits by repeated immunization with highly purified rat tonin (kindly supplied by Drs. G. Thibault and J. Genest, Canada) as previously described. As shown in Figure 1, the antitomin serum used for RIA (final dilution, 1:10,000) did not show any cross-reactivity with mouse SMG renin (kindly supplied by Dr. K. Poulsen, Denmark), rat urinary kallikrein (kindly supplied by Drs. G. Thibault and J. Genest), or porcine pancreatic kallikrein (Sigma Chemical, St. Louis, MO, USA), while the dilution curve generated by the medium in which dispersed rat SMG cells were incubated paralleled that of standard tonin. The antiserum did not cross-react with other serine proteases nor with polypeptide hormones in RIA. The RIA procedure for tonin has been described elsewhere. The minimum detectable amount of tonin was 25 pg/tube, and the intra-assay and interassay coefficients of variation were 10 and 15%, respectively.

The tonin activity of some incubation media was also determined by the amount of ANG II generated from ANG I, as previously reported. Before RIA for ANG II, 0.05-ml aliquots of samples were incubated with 3.9 X 10⁻⁸ M ANG I at 37°C for 60 minutes in a total volume of 1 ml of 50 mM phosphate buffer, pH 6.8, containing 0.1% bovine serum albumin, 8 mM 8-hydroxyquinoline, 1 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM 2,2'-dipyridyl.

Angiotensin II Radioimmunoassay

Angiotensin was determined by RIA using rabbit anti-ANG II serum (Mitsubishi Yuka, Tokyo, Japan). The antiserum against ANG II showed the following cross-reactivities: ANG I, 5.7%; ANG III, 73.9%; [Asn¹, Val⁵]ANG II, 126.9%; [des-Asn¹, Ile⁷]ANG II, less than 0.05%. The total volume of the incubation mixture was 0.3 ml and contained 0.1 ml of standard or sample, 0.2 ml of antibody (final dilution, 1:240,000), and 0.1 ml of ¹²⁵I-labeled ANG II (~5000 cpm; specific activity, 1550 μCi/μg; New England Nuclear, Boston, MA, USA), all diluted in 50 mM phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin. Incubation was carried out at 4°C for 48 hours. Separation of antibody-bound ¹²⁵I-labeled ANG II from free radioligand was accomplished by the double-antibody method. The minimum detectable amount of ANG II was 1 pg/tube. The intra-assay and interassay coefficients of variation were less than 10%. Since the antibody significantly cross-reacted with ANG I, the amount of ANG II generated was obtained by subtracting the values in the control tubes containing ANG I alone from those in the tubes in which samples and ANG I were coincubated.

Gel Exclusion Chromatography

For gel exclusion chromatography, a 0.9 X 58-cm column was packed with Sephadex G-100 fine resin (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated, and developed at 4°C with 50 mM phosphate buffer, pH 7.4, containing 77 mM NaCl (phosphate-buffered saline). Aliquots of incubation medium were loaded onto the column and eluted with the same buffer. The eluate was collected in fractions, and the radioactivity was determined in each fraction. The elution profile of the column was determined by adding 0.1 ml of ¹²⁵I-labeled ANG II (1000 cpm) to each fraction.

**Figure 1.** Rat tonin radioimmunoassay. Cross-reactivity of tonin antibody with rat urinary kallikrein, mouse submandibular gland (SMG) renin, or porcine pancreatic kallikrein (×). Dilation curve generated by incubation media (○) is compared with that of standard tonin (●). B/Bo = fraction of trace binding.
were applied to the column and eluted with phosphate-buffered saline at a flow rate of 8.6 ml/hr by descending flow; 0.4-ml fractions were collected.

Chemicals
The following agents were used for incubations: /-epinephrine, /-norepinephrine, /-isoproterenol, dl-phenylephrine, carbachol, histamine, serotonin, and N',O' dibutyryl cyclic AMP (all from Sigma); methyl isobutylxanthine (Calbiochem, La Jolla, CA, USA); ANG I, ANG II, and vasoactive intestinal peptide (Peptide Institute, Osaka, Japan); phenolamine (CIBA-Geigy, Basle, Switzerland); dl-propranolol (ICI, Manchester, England); A23187 (Eli Lilly, Indianapolis, IN, USA); diltiazem (Tanabe Pharmaceutical, Osaka, Japan); and nifedipine (Bayer Pharmaceutical, Leverkusen, West Germany). None of these drugs affected the viability of the dispersed cells or interfered with the RIA.

Statistics
Statistical significance of the differences between group means was tested using Student's t test. Values are presented as means ± SEM.

Results
There was a significant (p<0.01) correlation between values determined by RIA and those of tonin activity as measured by ANG U generation in the same samples; the coefficient of correlation (r) was 0.928 (n = 23). Basal release of immunoreactive tonin from dispersed SMG cells was 175 ± 12.2 ng/hr/10^6 cells (n = 10), while the tonin activity in terms of ANG II generated from ANG I was 24.6 ± 1.7 pmol/hr/10^6 cells. Therefore, the immunoreactive tonin released into the medium represents an active enzyme capable of cleaving ANG I into ANG II.

As shown in Table 1, both norepinephrine and epinephrine stimulated the release of tonin from dispersed SMG cells incubated in vitro. Phenylephrine, an /-adrenergic agonist, also significantly stimulated secretion of tonin in a dose-dependent manner (10^-5-10^-4 M; Figure 2), whereas isoproterenol, a /-adrenergic agonist, had no effect on its release (see Table 1). Phentolamine, an /-adrenergic antagonist, completely blocked norepinephrine-stimulated tonin release, while it had no effect on basal release (Figure 3). Propranolol, a /-adrenergic antagonist, failed to affect either basal or norepinephrine-induced tonin release (data not shown).

Removal of Ca^2+ from the incubation medium had little effect on basal tonin release (Figure 4). Whereas both norepinephrine (10^-3 M) and phenylephrine (10^-4 M) significantly stimulated tonin release in the presence of Ca^2+, removal of Ca^2+ from the incubation medium abolished their stimulatory effect (see Figure 4). Diltiazem (10^-4 M) and nifedipine (10^-4 M), which are selective calcium channel blockers, inhibited the norepinephrine-stimulated tonin release but had little effect on basal release (Figure 5). The divalent cation ionophore A23187 significantly stimulated the release of tonin in concentrations ranging between 5 and 10 μg/ml (Figure 6). This stimulatory effect was not observed in Ca^2+, Mg^2+ -free medium; however, A23187

### Table 1. Effects of Various Drugs on the Release of Immunoreactive Tonin from Dispersed Rat Submandibular Gland Cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Immunoreactive tonin released (ng/hr/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>167 ± 8</td>
</tr>
<tr>
<td>Norepinephrine (10^-4 M)</td>
<td>267 ± 8*</td>
</tr>
<tr>
<td>Epinephrine (10^-4 M)</td>
<td>328 ± 20*</td>
</tr>
<tr>
<td>Isoproterenol (10^-4 M)</td>
<td>179 ± 8</td>
</tr>
<tr>
<td>Carbachol (10^-4 M)</td>
<td>189 ± 8</td>
</tr>
<tr>
<td>Histamine (10^-4 M)</td>
<td>177 ± 9</td>
</tr>
<tr>
<td>Serotonin (10^-4 M)</td>
<td>180 ± 10</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP (10^-3 M)</td>
<td>180 ± 8</td>
</tr>
<tr>
<td>Methylisobutylxanthine (10^-3 M)</td>
<td>181 ± 7</td>
</tr>
</tbody>
</table>

*p<0.001, compared with control value.

**Figure 2.** Effect of phenylephrine on the release of immunoreactive tonin from dispersed rat submandibular gland cells (1x10^6 cells/ml) during a 60-minute incubation at 37°C in Krebs-Ringer bicarbonate buffer in the presence (■) or absence (□) of phenylephrine. Each column represents the mean of five incubations; bars indicate SEM. Single (p<0.05) and double (p<0.001) asterisks indicate the statistical significance of the differences.
Discussion

The present study used dispersed SMG cells of the male rat to show that the immunoreactive tonin secreted in vitro is predominantly stimulated by α-adrenergic agonists, not by β-adrenergic or cholinergic agents. These results are apparently in contrast to previous in vivo experiments in which β-adrenergic agonist stimulated release of tonin into saliva from rat SMG.5-6 Compared with the in vivo studies, in which only isoproterenol was used as a stimulus for tonin release,5-6 the present study clearly reveals that epinephrine and norepinephrine, physiological catecholamines, as well as phenylephrine, a pure α-adrenergic agonist, are potent releasers of tonin in vitro, an effect that was blocked by phentolamine but not by propranolol. In previous in vivo experiments, despite the stimulatory effect of theophylline, a phosphodiesterase inhibitor, on tonin release, dibutyryl cyclic AMP itself had no effect.6 Likewise, dibutyryl cyclic AMP and methylisobutylxanthine, which are potent, cell-permeable agents, had no effect on tonin release in the present in vitro studies.

The reason for this discrepancy is unknown. It may be partly accounted for by different assay methods (tonin activity vs RIA) or by different experimental designs (in vivo vs in vitro). Even though the tonin antibody used in this study did not cross-react with the various serine proteases tested, especially kallikrein, a possible cross-reactivity of the antibody with several nonkallikrein esterases in rat SMG10 cannot be ruled out. However, in the present study, the dilution curve of the incubation medium appeared to parallel that of standard tonin and tonin correlated well with tonin activity in the same incubation media. Furthermore, the secretory form of tonin in basal and norepinephrine-stimulated conditions was similar in molecular size to standard tonin. These data suggest but do not

Figure 3. Effect of phentolamine on norepinephrine-induced immunoreactive tonin release from dispersed rat submandibular gland cells (1 x 10⁶ cells/ml) incubated in the absence (○) and presence of phentolamine (●), norepinephrine (■), or both drugs (□). Each column represents the mean of five incubations; bars indicate SEM. Asterisk indicates significant difference (p < 0.01).

Figure 4. Effect of Ca²⁺ on α-agonist-induced immunoreactive tonin release from dispersed rat submandibular gland cells (4 x 10⁶ cells/ml) incubated in the absence (a) or presence (W) of 2.5 x 10⁻³ M Ca²⁺ and/or 10⁻⁵ M norepinephrine (NOREPI) and 10⁻⁴ M phenylephrine (PHENYL). Each column represents the mean of five incubations; bars indicate SEM. Asterisk indicates significant difference (p < 0.02).
prove that the tonin released into the medium is predominantly active.

The lack of in vitro secretory response to isoproterenol may be due to preferential destruction or perturbation of β-adrenergic receptors by proteolytic digestion because such alteration of receptor sensitivity following treatment with proteolytic enzymes has been reported.11,12 Nevertheless, the functional integrity of α-adrenergic receptors in the present study argues against this possibility. Under in vivo conditions, the neural tissue may release endogenous neurotransmitters, the pericellular elements may alter the secretory response, and the metabolic changes of injected secretagogues may affect the response. In accordance with our data are those of a recent in vitro study13 in which tonin activity was stimulated by norepinephrine but not by isoproterenol or methacholine from dispersed rat SMG cells. These in vitro data on tonin release are comparable to the stimulatory effect of α-adrenergic agonists on release of renin14-16 and kallikrein17,18 from rat SMG.

The present in vitro results also suggest that the stimulation of tonin release by α-adrenergic receptors is calcium-dependent, since α-adrenergic stimulation of tonin release was inhibited either by removal of Ca²⁺ from the medium or by the addition of selective calcium channel blockers. Furthermore, A23187, a divalent cation ionophore, mimicked the effect of α-adrenergic agonists in tonin release only when Ca²⁺ was present in the medium. This finding is also comparable to the α-adrenergic-mediated calcium dependency of renin13 and kallikrein13 release from the rodent SMG and suggests that activation of α-adrenergic receptors leads to Ca²⁺ flux into the cells, where it acts as an intracellular mediator of tonin release.

Like the renin in mouse SMG, the physiological importance of tonin in rat SMG is unknown. It has been suggested that tonin may be involved in the pathogenesis of one-kidney, one clip renovascular hypertension of animals.19,20 However, the involvement of tonin in experimental hypertension is controversial, as indicated by a recent report.21 Although it has been shown that tonin is secreted into the venous effluent of the perfused rat SMG,7 it seems unlikely that tonin plays an important role in the regulation of systemic blood pressure because of its complex formation with plasma α₁-macroglobulin, a potent inhibitor of tonin.22 On the other hand, tonin is secreted into saliva in large concentrations.4-6 Since it has been suggested that mouse SMG renin released into saliva by aggressive behavior may be transferred by bites from one animal to another,23,24 rat SMG tonin may likewise be related to fighting. It is also possible that tonin may be in-

![Figure 5](image-url)  
**Figure 5.** Effect of calcium channel blockers on norepinephrine-induced immunoreactive tonin release from dispersed rat submandibular gland cells (5 x 10⁶ cells/ml) incubated in the absence (■) and presence (■■) of 10⁻³ M norepinephrine and/or 10⁻⁴ M diltiazem and 10⁻⁴ M nifedipine. Each column represents the mean of five incubations; bars indicate SEM. Asterisk indicates significant difference (p<0.02).

![Figure 6](image-url)  
**Figure 6.** Effect of A23187 on the release of immunoreactive tonin from dispersed rat submandibular gland cells (5 x 10⁶ cells/ml) incubated in Krebs-Ringer bicarbonate buffer containing 2.5 x 10⁻³ M Ca²⁺ and 1.2 x 10⁻³ M Mg²⁺. Each column represents the mean of five incubations with (■) or without (■■) A23187; bars indicate SEM. Single (p<0.005) and double (p<0.001) asterisks indicate significant difference.

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volved in the processing of uncharacterized biologically active peptide (or peptides) in rat SMG, because
the γ subunit of nerve growth factor and epidermal growth factor–binding protein, which are arginine estero-
pitidases with 75% sequence homology to tonin,23 process pro-β–nerve growth factor26 and pro-epidermal
growth factor27 in mouse SMG, respectively.

Acknowledgments
We thank Drs. Jacques Genest and Gaétan Thibault, Clinical Research Institute of Montreal, Canada, for kindly supplying
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Figure 7. Effect of Ca2+ and Mg2+ on A23187-induced
immunoreactive tonin release from dispersed rat submandibular

gland cells (4 x 10^6 cells/ml) prepared in Ca2+-Mg2+-free
Krebs-Ringer bicarbonate buffer and incubated in the absence
(□) and presence (■) of A23187 (10 μg/ml). Either 2.5 x 10^-3 M
Ca2+ (□) or 1.2 x 10^-3 M Mg2+ (■) was added in combination
with the ionophore. Each column represents the mean of five
incubations; bars indicate SEM. Asterisk indicates significant
difference (p < 0.005).

Figure 8. Sephadex G-100 fine gel exclusion chromatography of incubation medium. Aliquots of the incubation
medium during the control period (A) and after stimulation by norepinephrine (B) were applied to a 0.9 x 58-cm
column and eluted with phosphate-buffered saline; 0.4-ml fractions were collected. Concentrations of immuno-
reactive tonin in fraction eluates are shown; open circle indicates undetectable concentration. Elution positions of

calibration standards are indicated at the top. Vo = void volume; BSA = bovine serum albumin; Ovalb = ovalbu-
min; Chymo = chymotrypsinogen A; Myo = myoglobin; Cytoc = cytochrome C; Vt = total volume.
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