A Potent Vasoconstrictor in the Rat Submandibular Gland

T. Yamaguchi, O.A. Carretero, and A.G. Scicli

We detected a novel vasoconstrictor in an arginine esterase fraction separated from fractions containing tonin and other esterases that were obtained from a rat submandibular gland extract. When tested on isolated rabbit aorta rings, the substance caused dose-related contractions that were slow in onset, long-lasting, and difficult to reverse by rinsing. The substance acts directly on vascular smooth muscle, since preincubation with plasma or intact endothelium is not required. The fact that the constrictor was destroyed by heat and incubation with pronase suggests that it is a protein. Molecular sieving indicates an estimated molecular weight of 24,000 Da. It has a neutral isoelectric point that is higher than the pI of tonin, from which it can be separated by anion exchange chromatography. A small amount of the vasoconstrictor was obtained by gel filtration and eluted from isoelectric focusing polyacrylamide gels. The purified substance showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was a potent vasoconstrictor; an estimated concentration of 2.5 nM induced contraction of isolated rabbit aorta rings ranging from 15% to 40% of the maximum contraction obtained by 60 mM KCl. Contraction was completely blocked by 1 mM (p-amidinophenyl)methanesulfonyl fluoride, a serine protease inhibitor. Contractile activity was not affected by hirudin, a thrombin inhibitor, but was completely inhibited by soybean trypsin inhibitor and blunted by aprotinin; thus it may be a trypsin-like serine protease. Purified vasoconstrictor preparation showed hydrolyzing activity on Pro-Phe-Arg-methylcoumarin amide, a kallikrein substrate. We conclude that a novel vasoconstrictor serine protease is present in the rat submandibular gland. (Hypertension 1991;17:101-106)

The presence of esterases (proteases) in mamalian salivary glands was recognized more than 60 years ago. Although proteolytic activity varies from species to species, the rat submandibular gland contains high concentrations of a number of proteases, among them glandular kallikrein, tonin, and related enzymes, only a few of which have been characterized in terms of their biological activity. Glandular kallikrein releases the vasodilator bradykinin from plasma precursors, whereas tonin releases the vasoconstrictor angiotensin II. Ability to induce formation of vasoactive peptides and evidence suggesting that they are released into the bloodstream led to the hypothesis that some of the compounds present in the submandibular gland are involved in local or systemic regulation of vascular resistance. The biological effect of glandular kallikrein and tonin on vascular contractility is secondary to their capability to release vasoactive peptides, although it has been reported that tonin may directly contract vascular tissue or potentiate the vasoconstrictor activity of norepinephrine in isolated vessels. Other proteases such as thrombin and trypsin also have a direct effect on vascular contractility. We separated out fractions from a rat submandibular gland homogenate based on their esterolytic activity and tested them for vasoconstrictor capability in isolated vascular tissue. In so doing, we have identified a novel protein vasoconstrictor that does not require blood components to contract isolated vascular tissue. It appears to be a different serine protease from kallikrein, tonin, and thrombin.

Methods

Acetylcholine, norepinephrine, hirudin, and rat thrombin were purchased from Sigma Chemical Co., St. Louis, Mo.; (p-amidinophenyl)methanesulfonyl fluoride (p-APMSF) from Calbiochem-Boehringer, La Jolla, Calif.; angiotensin II from Bachem, Torrance, Calif.; [3H]tosyl arginine methyl ester ([3H]TAME) from Amersham, Arlington Heights, Ill.; and Pro-Phe-
Arg-methylcoumarin amide (Pro-Phe-Arg-MCA) from Peninsula Laboratories, Belmont, Calif.

Fractionation of Rat Submandibular Gland Extract

Submandibular glands (45.2 g) were obtained from 60 male Sprague-Dawley rats weighing 300–350 g. The glands were homogenized and an extract was prepared as described previously. The extract was dialyzed against 0.1 M phosphate buffer (pH 7.0) containing 1 mM Na2EDTA and applied to a DEAE-Sephadex A-50 column equilibrated with the same buffer. The column was washed until A280 was below 0.005. Absorbed proteins and the vasoconstrictor fraction were eluted using a linear gradient of 0–0.15 M NaCl in equilibrating buffer. Under these conditions, proteases such as esterase and glandular kallikrein remained attached to the gel. Arginine esterases were monitored by measuring their esterolytic activity using [7H]TAME as a substrate at pH 8.0 and 30°C. Tritiated methanol is released by the enzymes in this method. TAME esterolytic activity was expressed as counts per minute (cpm) of 2H0-CH3 released per minute of incubation and per milliliter of incubation medium. Tonic activity was measured fluorometrically using angiotensin I as a substrate. Hydrolyzing activity on Pro-Phe-Arg-MCA, a synthetic kallikrein substrate, was measured using 50 mM Tris/HCl (pH 8.0) and 0.1 M NaCl at 0.02 mM substrate concentration. Generated 7-amino-4-methylcoumarin was determined fluorometrically. Proteins were measured according to Bradford’s method using bovine serum albumin as a standard. Vasoconstrictor activity was determined using rings isolated from a rabbit thoracic aorta. Male New Zealand rabbits weighing 1.5–2.0 kg were anesthetized with sodium pentobarbital (125 mg) and bled by severing the thoracic aorta. Blood was washed out with saline and rings measuring 3–5 mm were removed. The rings were attached to stainless steel wires connected to a Grass force displacement transducer (model 7D polygraph, Grass Instrument Co., Quincy, Mass.). They were then immersed in a 4-ml tissue bath maintained at 37°C and containing modified Krebs-Henseleit solution (mM: NaCl 118, KCl 4.6, NaHCO3 27.2, MgSO4 1.1, CaCl2 1.75, EDTA-Na2 0.03, d-glucose 11.1) bubbled with a 5% CO2–95% O2 mixture. The rings were stretched to a passive force of 1 g and allowed to reach equilibrium for 60–120 minutes, after which they were contracted with 60 mM KCl. After rinsing and a 30-minute reequilibration period, the vessels were challenged with up to 500 μl of each solution. Contraction expressed as a percentage of the maximum amount induced by 60 mM KCl. To eliminate the endothelium, the intima of the aortic ring was rubbed away with a cotton swab. Completeness of de-endothelialization was assessed by preconstricting the rings with 15 mM norepinephrine and demonstrating the absence of vasorelaxation in response to 1.4 μM acetylcholine, which induced full relaxation in precontracted intact vessels.

Physicochemical Characterization

Heat stability was studied by immersing the tube containing the constrictor in a boiling water bath for 10 minutes. To determine whether the constrictor was a protein, the sample (24.4 μg/250 μl) was shaken with 200 μl pronase in an immobilized agarose gel suspension (2.4 units/ml gel, Pierce, Rockford, Ill.) for 20 minutes at 25°C. Agarose gel suspension (Sepharose 4B, Pharmacia, Piscataway, N.J.) was used instead of pronase in control experiments. Both gels were equilibrated with Krebs-Henseleit solution. After incubation, gel suspensions were allowed to settle and 50 μl supernatant was assayed.

To estimate the molecular weight of the constrictor, fractions containing contractile activity were separated further by gel filtration on a Superose 12 HR 10/30 column (Pharmacia). Gel filtration was carried out with 0.1 M sodium phosphate buffer (pH 6.6) containing 0.15 M NaCl as a running buffer at a flow rate of 0.5 ml/min. The molecular weight of the constrictor was estimated from a calibration line prepared by passing standards having the following molecular weights through the same columns: bovine thyroglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000), myoglobin (17,000), ribonuclease A (13,700), and vitamin B12 (1,350). To estimate pl and obtain further purified material, the vasoconstrictor fraction from the gel filtration column was analyzed by isoelectric focusing on an Ampholine gel plate (pH 5.5–8.5, Pharmacia). After electrophoresis, the gel plates were sliced into 2-mm fractions. The slices were minced and macerated with distilled water and pH was measured at 4°C, after which each fraction was assayed for contractile activity. No changes in vascular tone were observed if slices were obtained from gel plates subject to isoelectric focusing without adding the sample (blank).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the methods previously mentioned with 12.5% gel.

Inhibitor Experiment

Effects of various protease inhibitors on the contractile activity of the substance were examined. The sample was preincubated with each protease inhibitor for 15 minutes at 25°C, after which the mixture was added to the organ bath and contractility was measured. The inhibitors by themselves have no effect on muscular contractility at the concentrations used.

Results

Figure 1 shows the separation of arginine esterases from the submandibular gland homogenate by anionic exchange chromatography during the salt gradient from 0 to 0.15 M. Three different TAME esterase peaks were identified. Tonic activity was eluted with the second and third esterase peaks. The first esterase peak was pooled as fraction II. Tonic activity was pooled with the second and third esterase peaks as fraction II. When tested for vasculotropic
activity using isolated aortic rings, fraction I induced a strong contractile response but fraction II did not, even with four times more protein than fraction I (Table 1). Contraction by fraction I showed a dose-related increase (Figure 2). Angiotensin I converting activity, which is an index of tonin enzyme activity, was also detected in fraction I, but the amount was only about 1/9 that of fraction II (Table 1).

The contraction induced by fraction I was slow in onset, taking around 10–15 minutes to reach a maximum. It was long-lasting and resistant to washing, since 12 washes (three changes of the chamber fluid per wash) were needed to return tension to near-baseline levels (Figure 3A). Intact rabbit aorta rings, which were precontracted with 15 nM norepinephrine, contracted further with cumulative addition of fraction I but relaxed with further addition of thrombin (Figure 3B). Direct addition of thrombin to norepinephrine-contracted rings resulted only in relaxation (Figure 3C). The contractile response to fraction I did not depend on the endothelial lining, since both intact and de-endothelialized vascular tissue contracted with similar potency, that is, 56.0±15.8% of maximum contraction in intact aortas (n=8) and 47.7±12.9% in denuded aortas (n=4) (NS by Student's t test).

Heating fraction I at 100°C for 10 minutes completely eliminated contractility. When filtered through a Superose 12 column, the contractile compound was eluted as a macromolecular substance with an estimated molecular weight of 24,000 Da. Table 2 shows the effects of some protease inhibitors on the contractile activity of fraction I. Hirudin, a specific thrombin inhibitor, blocked the clotting activity of rat thrombin (0.8 μg/ml) even at a low concentration (10 units/ml) but did not affect the contractions induced by fraction I. Contractile activity was blocked by serine protease inhibitors, partially by aprotinin, and completely by soybean trypsin inhibitor (SBTI) and p-APMSF.

SDS-PAGE analysis of fraction I is shown in Figure 4A. Fraction I was found to contain several protein bands. Small-scale purification was attempted by gel filtration on Superose 12 and isoelectric focusing on precast polyacrylamide gel. The isoelectric point of the vasoconstrictor activity was

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**Figure 1.** Graph showing DEAE-Sephadex A-50 chromatography of rat submandibular gland extract. After loading, fractions were eluted by a 0–0.15 M NaCl linear gradient in 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA. Fractions were tested for esterolytic activity on [3H]tosyl arginine methyl ester ([3H]TAME) and two pools were made, one containing the fractions identified as the first esterase peak (I, fraction I) and the other obtained by pooling the fractions containing tonin and the second and third esterase peaks (II, fraction II). Each pool was thoroughly dialyzed against 0.1 M phosphate buffer-0.15 M NaCl (pH 7.0) and tested for contractile potency on isolated rabbit aortic rings.

**Figure 2.** Line graph showing dose-related contractions induced by fraction I. Contractile activity was expressed as the percentage of maximum contraction obtained with 60 mM KCl.

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**Table 1.** Contractile and Tonin Activity in Fractions I and II Obtained by DEAE-Sephadex A-50 Chromatography of Rat Submandibular Gland Homogenate

<table>
<thead>
<tr>
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<th>Fraction I</th>
<th>Fraction II</th>
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<tbody>
<tr>
<td>Contractile activity (%)</td>
<td>64 (7.3 μg)</td>
<td>0 (28 μg)</td>
</tr>
<tr>
<td>Tonin activity (nmol His-Leu/mg)</td>
<td>51.5</td>
<td>452.8</td>
</tr>
</tbody>
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Contractile activity induced by the fraction (μg protein between parentheses) was expressed as percent maximum contraction with 60 mM KCl. Tonin activity was determined by measuring the generation of His-Leu from angiotensin I.
determined to be 7.3. When this fraction was analyzed by SDS-PAGE, only a single protein band was observed (Figure 4B). It had a molecular weight estimated at 27,500 Da, slightly higher than the one calculated by molecular sieving. The final vasoconstrictor preparation (0.3 μg protein, 2.5 mM in organ chamber) elicited 15–40% of the maximum contraction of 60 mM KCl (n=8). The purified vasoconstrictor induced slow, long-lasting, rinse-resistant contractions that were sensitive to p-APMSF and SBTI and resistant to hirudin, similar to those induced by fraction I, the crude preparation (data not shown). The purified vasoconstrictor preparation hydrolyzed Pro-Phe-Arg-MCA, a kallikrein substrate, at a rate of 11.2 nmol/min/mg.

**Discussion**

A fraction of the rat submandibular gland separated out by anionic exchange chromatography was found to contain a potent and novel vasoconstrictor, most likely an enzyme. The fact that this substance was inactivated by heat as well as by digestion with the nonspecific peptidase pronase, together with its molecular weight (24,000–27,500 Da), indicates that it is a protein. Furthermore, partial inactivation with aprotinin and complete inhibition with SBTI and p-APMSF, a general serine protease inhibitor, suggest that it is a serine protease. Although the vasoconstrictor may be a protease, preincubation with

**Table 2. Effects of Protease Inhibitors on Contractile Activity of Fraction I**

<table>
<thead>
<tr>
<th>Sample + inhibitor</th>
<th>Contractile activity (%)</th>
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<tbody>
<tr>
<td>Sample + saline</td>
<td>100</td>
</tr>
<tr>
<td>Sample + p-APMSF</td>
<td>0 (n=4)</td>
</tr>
<tr>
<td>Sample + SBTI</td>
<td>0 (n=6)</td>
</tr>
<tr>
<td>Sample + aprotinin</td>
<td>68.6±14.2 (n=4)</td>
</tr>
<tr>
<td>Sample + hirudin</td>
<td>94.2±42.0 (n=6)</td>
</tr>
</tbody>
</table>

The volume of fraction I that induced a 1.68±0.44 g increase in tension (n=6) was incubated with each inhibitor at the concentrations shown between parentheses for 15 minutes at 25°C, and the contractile activity of the mixture was measured. The effect of the inhibitors is shown as percent activity of control samples incubated with saline. Values are mean±SD. n, Number of experiments; p-APMSF, (p-aminophenyl)methanesulfonyl fluoride; SBTI, soybean trypsin inhibitor.

**Figure 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of rat submandibular gland vasoconstrictor fractions.** A: Fraction I (3 μg). B: Purified vasoconstrictor preparation (0.2 μg). Molecular weight standards were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), SBTI (20.1 kDa), and α-lactalbumin (14.4 kDa). Protein bands were stained by silver nitrate.
plasma was not necessary for activity; nor is it likely that the aortic rings contained enough residual plasma to provide a substrate from which a vasoconstrictor could be released, since they were rinsed thoroughly. Thus vasoconstriction could not be due to the release of angiotensin II by tonin as a contaminant of residual plasma, because fraction II, which contained almost nine times more tonin than fraction I, was inactive. This also implies that the contractility of fraction I is not due to direct vasoconstrictive action of tonin as a contaminant. In preliminary experiments, we failed to show vasoconstrictor activity when the fluid-bathing the rings during the contractile phase was boiled for 10 minutes and reapplied to fresh, uncontracted rings. Thus the vasoconstrictor appears to act directly on the vessel wall.

Bovine and porcine thrombins, serine proteases that are also arginine esterases, are reported to have potent vasodilating action; however, they can also contract vascular smooth muscle directly at high concentrations. The contractile compound in fraction I did not resemble rat thrombin, which only served to clearly identify this enzyme but also to study the mechanism whereby it causes vascular smooth muscle to contract.

At present we can only speculate about the identity of the novel and potent vasoconstrictor described here. The rat submandibular gland contains a number of arginine esterases. A few of them have been well characterized, such as glandular kallikrein, tonin, esterase B, and esterase γ. Under our experimental conditions, kallikrein and esterase γ should be retained by the DEAE-Sephadex A-50 column. Both contractile and Pro-Phe-Arg-MCA activity are found in a purified protein (pl = 7.3) that is clearly separable from tonin (pl = 6.2) as well as from a third esterase, which we have previously named esterase B (pl = 5.6). Characteristics of other submandibular gland esterases are still unknown. All of them belong to a group of immunologically related serine proteases coded by a set of closely associated genes named the kallikrein gene family. The rat has 8–17 kallikrein genes, many of which are expressed in the submandibular gland. They are serine proteases and most are arginine esterases, a property common to all trypsin-like enzymes. Thus, it is possible that the vasoconstrictor protease is an as-yet-unidentified member of the kallikrein family. Further studies are needed to confirm or refute this hypothesis. Large-scale purification is necessary not only to clearly identify this enzyme but also to study the mechanism whereby it causes vascular smooth muscle to contract.

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