Discharge Effect on Pancreatic Exocrine Secretion Produced by Toxins Purified from Tityus serrulatus Scorpion Venom*

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Three toxic polypeptides were purified from the venom of the Brazilian scorpion Tityus serrulatus by means of gel filtration in Sephadex G-50 and ion-exchange chromatography in carboxymethylcellulose. The peptides are basic molecules with molecular weights in the range of 7000 for which the amino acid compositions and sequences were determined. The effect of the purified peptides on pancreatic exocrine secretion in the guinea pig was studied. Biochemical measurements show that the cells are stimulated by these peptides to discharge their zymogen granules. Light and electron microscopic images confirm the biochemical measurements. At the light microscope level, acinar cells show dramatically fewer zymogen granules than in control pancreas with the appearance of large vacuoles and some loss of morphological integrity. Electron micrographs display apical regions devoid of zymogen granules and condensing vacuoles whereas acinar lumina contain crystalline secretory material. The secretory effect observed in vitro is comparable to that of carbachol and that of the peptidergic secretagogue cholecystokinin-pancreozymin.

The first report on the isolation of a toxic component, named Tityustoxin, from the venom of the scorpion Tityus serrulatus Lutz and Mello, was published in 1966 (13). Subsequently, Toledo and Neves (36) and our group (27, 40) have demonstrated the presence of more than one toxic component in the same venom. In fact, the component named gamma was the first toxin from Brazilian scorpion venoms for which the NH₂-terminal amino acid sequence was determined (27). Further chemical characterization of the toxins from T. serrulatus described (5, 28, 30). The specificity and affinity of toxin gamma for Na⁺ channels (3, 39) and the effect of toxin II-9 on voltage-dependent K⁺ channels of squid axon membranes were also documented (7, 29). A summary of the known amino acid sequences of various toxic peptides from T. serrulatus was reviewed by Possani in 1984 (28). Briefly, the procedure includes size-exclusion chromatography and two consecutive steps of weak cation-exchange chromatography on carboxymethylcellulose (CM-cellulose). The first separation was performed in a column (0.9 X 200 cm) of Sephadex G-50 (medium) equilibrated with 20 mM ammonium acetate buffer, pH 4.7, from which four main fractions were obtained. Each of the toxic fractions (II to IV) were further separated on CM-cellulose at two different pH values. The first CN-cellulose column (0.9 X 30 cm) was developed in ammonium acetate buffer, pH 4.7. In this step, toxic components II-11, III-8, III-10, and IV-5 were separated, corresponding to the main toxic components of the venom (28). Each of these components was subsequently purified by rechromatography of the dialyzed fractions through a second CM-cellulose column equilibrated with 50 mM sodium phosphate buffer, pH 6.0. A linear gradient from 0-0.5 M NaCl in the same buffer was used to elute the toxic proteins in homogeneous form.

Enzymatic digestion mixtures of reduced and alkylated proteins were separated into their component peptides by reverse-phase high performance liquid chromatography (HPLC) on octadecylsilane columns developed with linear gradients of 0.1% trifluoroacetic acid and integrated with up to 60% acetonitrile. Isolated fractions were evaporated to dryness under a stream of nitrogen for solvent removal prior to amino acid analysis and sequenator application.

Lethality Tests—Lethality tests in mice were conducted as described (27). Basically, aliquots were injected intraperitoneally into 20-g mice that were observed for toxic effects. Among the symptoms of toxicity are hyperexcitability, lacrimation, apnea, and partial paralysis of the rear limbs, diarrhea, respiratory failure, and death. Acutely intoxicated animals were usually killed according to approved animal protocols before they died of the toxin.

Amino Acid Composition and Sequence Determination—Amino acid compositions of the three toxins purified from Tityus serrulatus venom were determined (27). Portions of this paper (including part of "Materials and Methods" and Figs. 2, 4, and 13) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

The abbreviations used are: HPLC, high performance liquid chromatography; KRB, Krebs-Ringer bicarbonate.

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acid compositions were obtained with acid hydrolysates of purified proteins analyzed with a Durum/Dionex D-502 automatic amino acid analyzer, as described (27). Prior to amino acid sequence determination, the toxins were either reduced and carboxymethylated or pyridylethylated (27, 28, 30). Determination of half-cystine was obtained after performic acid oxidation (23). Tryptophan content was deduced from sequence data.

Carboxypeptidase digestion for the determination of carboxy-terminal amino acid residues was carried out in 50 mM sodium acetate buffer, pH 3.7, with carboxypeptidase P. Release of free amino acids, and as a function of time, was determined by amino acid analysis directly from aliquots of the hydrolisate mixture. Usually, 10 nmol of reduced and carboxymethylated toxins (both IIII-11 and IV-5) were incubated with enzyme (5% of peptide concentration) at room temperature in Krebs-Ringer bicarbonate buffer, pH 7.2, equilibrated with 95% O2, 5% CO2 and supplemented with Eagle’s essential and non-essential amino acids, 14 mM glucose, 0.01% soybean trypsin inhibitor, and 0.2% bovine serum albumin (8).

Measurement of Exocrine Secretory Discharge—Pancreatic lobules were prepared according to the method described by Scheele and Palade (35), mounted on nylon monofilament discs, and preincubated for 10 min at 0°C. They were pulse-labeled for 10 min at 37°C with 0.4 μM [1,4,5-3H]t-leucine (10 μCi/ml) in KRB, then washed with 4.0 mM l-leucine KRB (KRB-chase). Upon transfer to chase incubation medium based on the total amylase distribution in pancreas and homogenized in water. Samples from incubation medium and tissue homogenates were prepared immediately for the radioactivity assay (15) in Aquasol-2 and counted in a Beckman LS 7000 liquid scintillation counter. Exocrine protein discharge was expressed as the percentage of proteins recovered from the incubation medium. Amylase activity was measured according to the Bernfeld method (6) and expressed as milligrams of maltose liberated in 15 min at 30°C. Results were expressed as above, i.e. the percentage of amylase released in the medium based on the total amylase distribution in tissue and incubation medium.

Measurement of lactate dehydrogenase activity was carried out on aliquots of the same incubation media to determine the extent of damage to cellular membranes. The spectrophotometric method of Reeves and Fimognari (31) was used.

RESULTS

Purification of Venom Protein Secretagogues—For the isolation of the venom secretagogue proteins we have used essentially the same procedure as described before (28, 30) except that an additional chromatographic step on CM-cellulose equilibrated and run in 50 mM sodium phosphate buffer, pH 6.0, was included. This step improves the quality of the purified toxins, eliminating possible minor contaminants still present after the initial ion-exchange separation. The purity of toxins was verified by electrophoretic mobility using the β-alanine/acetate urea gel system of Reisfeld et al. (62), as shown in Figs. 1 and 13. Amino acid composition and NH2-terminal amino acid sequence determination were additional criteria for assessing the purity of the secretagogue toxins, as described next.

Chemical Characterization of Toxins—The amino acid compositions in Table I show that the toxins are very similar peptides with respect to their molecular weights, half-cystine content, and high percentage of basic amino acid residues. Components II-11 and IIII-10 have identical amino acid compositions. The Sephadex G-50 column (28) does not entirely separate fraction II from fraction IIII. These fractions are...
further resolved on CM-cellulose at pH 4.7 (28). The chromatographic profiles of toxin II-11 of fraction II and III-10 of fraction III are eluted at the same ionic strength. This indicates that, on the basis of chromatographic behavior and amino acid composition, these two components are indistinguishable and confirm our original findings (27, 28). Also, the amino acid sequences of both components (II-11 and III-10), as shown next, are identical. Furthermore, these sequences are not distinguishable from that of toxin gamma previously described (5, 28). For these reasons, we conclude that toxins II-11, III-10, and gamma correspond to the same venom component. Toxin III-8 has 62 amino acid residues whereas toxin IV-5 has 60, which correspond to molecular weights of 6957 and 6918, respectively. However, their amino acid sequences are distinct (Fig. 2, A and B, of the Miniprint) and also different from toxin gamma, as shown in Fig. 3. The complete amino acid sequence of toxin III-8 is shown in Fig. 2A; Fig. 2B shows the sequence for toxin IV-5. These sequences were obtained by automated Edman degradation procedures (9) on the whole proteins following reduction and alkylation of the half-cystine residues (27, 28) and by sequence determination of peptides generated by enzymatic cleavage, as indicated in the legends to Figs. 2 and 4 of the Miniprint. Fig. 4 shows the separation of some of the cleaved peptides by reverse-phase HPLC. Small differences also occur between the amino acid compositions shown in Table I and the sequences reported in Fig. 3. The amino acid sequence determined for toxin IV-5 has 1 tyrosine residue more than that for a tyrosine in toxin 111-8. Since the half-cystine content was obtained as cysteic acid, after oxidation with performic acid (variable recoveries of 65–95%) the values for half-cystine were taken from amino acid sequence data. The COOH-terminal amino acids cystine and lysine were determined from analyses obtained from a time course hydrolysis of reduced and carboxymethylated toxins (III-8 and IV-5). Although the partial (28) and the full amino acid sequences for toxin III-10 (30) were reported earlier, its sequence determination was repeated in the present work (data not shown).

**Sequence Comparisons—**In Fig. 3, we compare the sequences of three major toxins from the venom of *T. serrulatus*. In comparing these amino acid sequences, artificial gaps (represented by series of dashes) were introduced to enhance similarities. The numbers above the sequences show the 65 possible positions. Boxes enclose areas in which the amino acids are identical. Although a minor segment of the COOH-terminal region of toxin IV-5 is still unknown, we have included the expected amino acids, based on the amino acid composition shown in Table I.

Close inspection of these sequences shows that only 21 amino acids are located in identical positions in all three sequences. If we compare toxin gamma with III-8, there is 69% similarity (49 amino acid residues out of 65 total), whereas toxin gamma compared with IV-5 shows 38% identity (25 out of 65). Comparison of toxin III-8 with IV-5 shows only 34% identity (22 out of 65). The most conserved parts of the sequences are located in three segments: the NH2-terminal sequence (25 out of 65). Comparison of toxin III-8 with IV-5 shows only 34% identity (22 out of 65). The most conserved parts of the sequences are located in three segments: the NH2-terminal region, positions 1–4, where several amino acids are identical and conservative substitutions occur between glutamic and aspartic acids; the middle of the sequence, between positions 35 and 40; and between amino acid positions 45 and 50. Gaps were placed at positions 0, 29, and 42–43 and in the COOH-terminal region of toxin IV-5.

**Direct Secretory Effect of Toxins on Pancreatic Lobules—**We determined that venom fractions that were lethal in mice were also the most potent secretagogues in guinea pig pancreatic lobule pulse-chase experiments (10, unpublished data). Therefore, our attention in purification and biochemical characterization of venom proteins for studies of pancreatic secretion was focused on similar fractions.

**In vitro** dose-response experiments showed that pancreatic lobules respond to stimulation of exocrine discharge by the three purified *T. serrulatus* toxins at levels comparable with the effect elicited by the secretagogue carbachololine. In Fig. 5, a dose of 10⁻⁸ M is seen as the level at which all three toxins begin to cause a discharge effect. Optimum levels of discharge are achieved at different concentrations for the three toxins, with toxin III-10 showing the highest degree of potency at 10⁻⁸ M, toxin IV-5 at 10⁻⁷ M, and toxin III-8 at 10⁻⁶ M. At the lowest toxin concentration of 10⁻¹⁰ M, secretagogue activity drops to control levels of 5–7% discharge of ³H-secretory proteins. Each of the three toxins produces a characteristic dose-response curve for discharge effect, with toxins III-8 and IV-5 producing a greater stimulation of pancreatic secretion than did toxin III-10 at the two highest dose levels. Maximal stimulation of 47% ³H-protein discharge is achieved by toxin III-10 at a lower dose (10⁻⁸ M), but it never elicits the higher levels of stimulation, 58 and 54%, produced by toxins III-8 and IV-5, respectively. Although these values are not statistically different at the 95% confidence interval, these differences are reproducible from experiment to experiment. When more toxin III-10 is present, the secretagogue effect is diminished and does not return to maximal stimulation at the highest dose of 10⁻⁶ M. We have tentatively identified this portion of the dose-response curve as the hypersecretory limb. These results were consistent through repeated experiments (see the legend to Fig. 5A). In
averaged 50% of the total. The dose-response curve for carbachol (not shown) also demonstrates a hypersecretory limb similar to that determined for toxin III-10.

Measurement of lactate dehydrogenase activity as an indicator of membrane integrity showed that no leakage of this cytosolic marker enzyme occurred during incubation with the toxins (data not shown). Thus, the secretagogue effects presented here are due to a true stimulation of the exocrine discharge mechanism mediated by the action of each of the toxins on pancreatic acinar cells.

**Light Microscopy Images**

**Fig. 6.** Light micrographs of guinea pig pancreatic lobules. Lobules were incubated in vitro for 3 h at 37°C under the following conditions: a, KRB alone; b, toxin III-8; c, toxin III-10; d, toxin IV-5 (all at $10^{-8}$ M). In Fig. 6a, unstimulated control pancreas is rich in darkly stained zymogen granules. The obvious effect of toxin stimulation is seen here as depletion of zymogen granules in panels b–d. Arrows indicate sites of vacuole formation. The micrographs are representative of two separate experiments. Bar = 10 μm.

enzymatic activity assays performed in parallel with the radiolabeled secretory protein measurements, the dose-response curves for amylase discharge in Fig. 5B retained the same unique features for each of the three toxins. Levels of amylase discharge, however, were never greater than 43% even at the highest doses of toxins.

All experimental discharge assays included measurements of activity stimulated by the muscarinic agonist carbachol, which is an analog of the natural effector acetylcholine. Carbachol has an advantage over acetylcholine in that its effects do not diminish as a result of the action of acetylcholinesterase. Carbachol at $10^{-5}$ M stimulated an average of 58% discharge of the pulse-labeled proteins. Carbachol-stimulated amylase activity secretory discharge
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Base-line secretion levels averaged 7%. When exposed to toxin III-8 at optimal levels of stimulation, the acini in Fig. 6c have lost a large number of zymogen granules with a discharge of labeled secretory protein at 58%. The "scalloped" surfaces of basal areas seen in Fig. 6a are no longer present, and acini seem to be flattened against one another with little or no distinct space between them. In Fig. 6d, under similar stimulation conditions, but with toxin III-10, zymogen granules are either reduced in number or even absent in some acinar cells in accordance with a 54% secretion of labeled proteins. This lobule portion has retained some of the distinctive basal areas of normal tissue but has developed some large vacuoles (arrows) in the Golgi and basal regions not seen in control specimens. The same is true for Fig. 6d in which tissue has been incubated with toxin IV-5 with a discharge activity of 60% of labeled proteins secreted. All acinar cells shown have some depletion of zymogen granules, and large intracellular vacuoles have appeared in many. All three toxins were as potent as carbamylcholine in their secretagogue effect in vitro. In this experiment, carbamylcholine-evoked release of labeled proteins was 60%.

The secretagogue effect of whole T. serrulatus venom on pancreas in vivo is dramatic, as shown in Fig. 7. In tissue removed at death, most acini have completely discharged their zymogen granules, whereas in those acini retaining granules, the numbers are greatly reduced. No newly formed granules are apparent in the Golgi area, although condensing vacuoles may be present but not visible at the light microscope level.

Electron Microscopic Images of Toxin-treated Pancreatic Lobules—In Fig. 8, portions of four cells are shown from an acinus in control tissue incubated for 3 h in vitro. The apical region is densely packed with mature zymogen granules, and the acinar lumen is compressed with numerous microvilli. Discharge of secretory enzymes has not obviously occurred because of the lack of content in this lumen. As seen at the light microscope level, the cells have retained their structural integrity. The lobules in Figs. 9 and 10 have been incubated with toxin III-8 but are from different specimen blocks of tissue. Most of the zymogen granules in Fig. 9 are discharged, but a number of condensing vacuoles in different stages of maturity remain in the two large Golgi complexes close to the apical plasmalemma. Present in the lumen are both secretory material and membranous debris. The luminal surface shows partial effacement, with the loss of microvilli on part of its acinar plasmalemma and on the plasmalemma of a centroacinar cell. A large acinar lumen in Fig. 10 is distended with both crystalline and fibrous secretory material and with detritus mostly in vesicular form. Microvilli are again absent on some plasmalemmal surfaces. Endoplasmic reticulum cister-
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Figure 11. Electron micrograph of pancreatic exocrine cells from a lobule incubated with 10^{-6} M toxin III-10. The Golgi complex (G) is disrupted and the number of zymogen granules is reduced. Bar = 1 \mu m.

The amino acid sequences of Figs. 2, A and B, and 3 show that these toxins have some similarities in their primary structures. Some gaps have to be included to align the half-cystine residues in the same relative positions. It has been suggested that, if the tertiary structure of toxins is preserved by maintaining the position of the disulfide bridges and the \( \alpha \)-helix and \( \beta \)-pleated sheet regions, then the same physiological effects are more likely to be expressed (22, 26, 30). This seems to be the case for the toxins of the Brazilian scorpion \textit{T. serrulatus}. The X-ray diffraction studies conducted with variant 3 from \textit{Centruroides sculpturatus} (11), a North American scorpion, and with toxin II from \textit{Androctonus australis} Hector, a North African scorpion (12), show that the most prominent structural features highly conserved in these toxins are an \( \alpha \)-helical region comprising residues 19–28, a three-stranded antiparallel \( \beta \)-sheet containing residues 2–4, 32–37, and 45–51, and three of the four disulfide bridges. The comparison shown in Fig. 3 suggests that similar identical amino acid sequences are conserved in toxins from \textit{T. serrulatus}. Further similarities among these toxins are shown by our results on the secretory effect of these toxic peptides in vitro.

Comparisons between control and toxin-stimulated tissue, as described here, reflect qualitative differences. The purpose of this paper is not to present evidence for morphological changes leading to pancreatitis but to verify the potential of the toxins as secretagogues. However, references to some other than the discharge of zymogen granules show that there may indeed be instances in toxin-treated tissue of early events related to acinar cell damage leading to the development of pancreatitis. In three of the very few electron microscope studies presented on clinically diagnosed human acute pancreatitis (2, 14, 18), there are manifestations of dilated acinar lumina with some loss of microvilli, the development of intracellular vacuoles, and the presence of intracellular debris and degenerated organelles in lumina. These cellular events also develop during the course of induced interstitial pancreatitis in the rat with hyperstimulatory doses of the peptide secretagogue caerulein (1, 19). Our initial interests in scorpion toxin-mediated pancreatic secretion were based on reports by Waterman (38) and Bartholomew (4), who found that patients stung by the Trinidadian scorpion \textit{Tityus trinitatis} developed clinical presentations of acute pancreatitis. Machado and da Silveira (21) were able to induce characteristic pathological lesions of acute pancreatitis in dogs administered sublethal doses of \textit{T. serrulatus} whole venom. Further investigations in this laboratory on the role of \textit{Tityus} venoms in the causation of pancreatitis must be based on knowledge concerning the mechanism of action of the component protein toxins.

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Toxin gamma has been characterized in our work with isolated giant squid axon as a reversible and specific blocker of the voltage-dependent sodium channel (7). Similarly, the effect of toxin gamma on sodium channels of the heart has been described (3, 17, 39). The two major types of ion channels known to exist in pancreatic acinar cells at this time are a Ca\(^{2+}\)-activated nonselective monovalent cation channel (K\(^{+}\)-permeable) and a Ca\(^{2+}\)-activated K\(^{+}\)-selective channel (24, 25).

Voltage-activated Na\(^{+}\) channels have been studied in insulin secreting cells (34) but have not yet been studied or demonstrated in acinar cells. Whether or not the toxins act directly on acinar cell receptors or ion channels, or indirectly through neuronal stimulation, is of interest in our understanding of how the toxins affect secretory discharge processes. If the secretagogue effect is indeed mediated by a direct modification of ion channel permeability, it is conceivable that this channel would be selective for sodium ions in a way similar to the effects already described in other systems (3, 7, 17, 39).

We are presently investigating these possibilities.
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Fig. 1A: Amino acid sequence of toxin I-8. 

Primary Structure determinations were carried out by automatic Edman degradation with results of each cycle above the arrows that indicate the order of determination. Structural determinations required to develop continuous sequences are indicated by letters before arrow sequences that are indicative of continuous determinations. D indicates determinations carried out with whole protein; N, for Asp-N peptides; T, for Tryptic peptides; and P, for Carboxypeptidase P timed hydrolysis. See Fig. 4 for separations of some of the enzymatic cleavages.

Fig. 2A: Amino acid sequence of toxin III-9. Primary structure determinations were carried out by automatic Edman degradation with results of each cycle above the arrows that indicate the order of determination. Structural determinations required to develop continuous sequences are indicated by letters before arrow sequences that are indicative of continuous determinations. D indicates determinations carried out with whole protein; N, for Asp-N peptides; T, for Tryptic peptides; and P, for Carboxypeptidase P timed hydrolysis. See Fig. 4 for separations of some of the enzymatic cleavages.

Fig. 2B: Amino acid sequence of toxin IV-5. The various methods used for determining this sequence are indicated as in Fig. 2A. The amino acids from the C-terminal segment for which the sequence is not yet deduced by difference between that of the amino acid composition known is marked by U and separated with commas. The content was (Table I) and the primary sequence determinations as indicated here.

Figure 3. Spectrophotometric scans of acid-urea Polyacrylamide gel electrophoresis (PAGE) of Scorpion Antivenom toxin run in 4 mm tube gels stained in Coomassie Blue. A = IV-5, B = III-9, C = III-10. 15 µg samples of each.

Figure 1. PRIMARY STRUCTURE OF TOXIN I-8 (FROM I. MILIARIS)