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CONTROL OF VENOM PRODUCTION AND SECRETION BY SYMPATHETIC OUTFLOW IN THE SNAKE *BOTHROPS JARARACA*

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Summary

Many studies have examined the morphological and biochemical changes in the secretory epithelium of snake venom glands after a bite or milking. However, the mechanisms of venom production and secretion are not yet well understood. The present study was undertaken to evaluate the role of the sympathetic nervous system in the control of venom production and secretion.

Venom glands were obtained from *Bothrops jararaca* (Viperidae) snakes, either unmilked previously or milked 4, 7 or 15 days before they were killed. Levels of tyrosine-hydroxylase-like immunoreactivity were higher in venom glands collected 4 days after milking, coinciding with the maximal synthetic activity of the secretory cells. The only catecholamine detected by high-performance liquid chromatography was noradrenaline, indicating the presence of noradrenergic fibres in these glands. In reserpine-treated milked snakes, no venom could be collected, and electron microscopic analysis showed narrow rough endoplasmic reticulum cisternae, instead of wide cisternae, and less well-developed Golgi apparatus compared with milked untreated snakes, indicating impairment of protein synthesis and secretion. The administration of isoprenaline or phenylephrine (β- and α-adrenoceptor agonists, respectively) to reserpine-treated milked snakes promoted the widening of the rough endoplasmic reticulum and restored venom production, but only phenylephrine restored the development of the Golgi apparatus and the formation of many secretory vesicles.

These results provide the first evidence that the sympathetic nervous system plays an important role in venom production and secretion in the venom glands of *Bothrops jararaca*. Understanding the importance of noradrenergic stimulation in venom production may provide new insights for research into the treatment of snakebites.

Key words: snake, *Bothrops jararaca*, venom production, venom secretion, venom gland, sympathetic innervation, adrenoceptors.

Introduction

Bothrops jararaca, a Brazilian solenoglyphous venomous snake, belongs to the subfamily Crotalinae, family Viperidae, and is responsible for most snakebite accidents that occur in the southeastern region of Brazil (Cardoso *et al.* 1993).

Venom glands of viperid snakes are related to salivary glands (Kochva and Gans, 1964, 1965), and their structure has been extensively studied. The venom produced in these glands is accumulated in a large central lumen (Kochva, 1960, 1987; Warshawsky *et al.* 1973; Mackessy, 1991) and, after manual extraction (milking) or after a bite, the secretory epithelium of the venom glands undergoes morphological and biochemical changes. The epithelial cells change from a cuboidal to a columnar shape, the rough endoplasmic reticulum (RER) cisternae expand, and venom is synthesized. The maximal synthetic activity of the secretory cells and the highest mRNA concentration are observed 4–8 days after milking; later, the synthetic activity decreases and the venom is gradually accumulated in the gland lumen, while the epithelium returns to a quiescent state (Ben-Shaul *et al.* 1971; Rotenberg *et al.* 1971; Oron and Bdolah, 1973; De Lucca *et al.* 1974; Kochva, 1978; Carneiro *et al.* 1991; Salomão, 1991). This cycle of venom production is long in comparison with that of mammalian salivary glands and secretion by the pancreas (Jamielson and Palade, 1967*a*,*b*; Amsterdam *et al.* 1969).

The control mechanisms involved in the regulation of venom synthesis and secretion in the venom glands are not understood. As the severing of the main nerve supply to the venom glands does not affect venom production, protein concentration or enzyme activity, it has been suggested previously that venom production is not under nervous control (Kochva, 1978). However, ultrastructural studies in the venom glands of the elapid snakes *Maticora birvirgata* and *Lapemis curtus* showed

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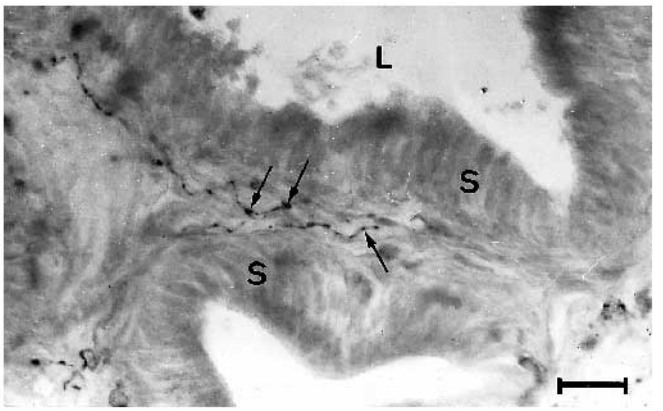

Fig. 1. Positive immunoreaction for tyrosine hydroxylase (arrows) in the intertubular space, near the secretory cells in a *Bothrops jararaca* venom gland, indicating the presence of catecholaminergic innervation. S, secretory cells; L, tubular lumen. Scale bar, 20 µm.

the presence of nerve terminals in close apposition to the secretory cell basal membranes (Gopalakrishnakone and Kochva, 1990, 1993). Moreover, it has been shown recently that chronic isoproterenol treatment modifies the protein profile of the venom and of the venom gland proteins in *Bothrops jararaca* (Nuñez-Burgos *et al.* 1993). Since it is known that the sympathetic system plays an important role in protein synthesis in mammalian salivary glands, to which the snake venom glands are related, the aim of the present study was to investigate the presence of sympathetic innervation and its possible participation in venom production and secretion by the venom gland of the snake *Bothrops jararaca*.

Materials and methods

Animals and venom glands

Adult *Bothrops jararaca* (Wied) of both sexes (*N*=46), weighing 140–240 g, were captured from the wild, identified by the Herpetology Laboratory from Instituto Butantan, and treated and kept as described by Breno *et al.* (1990). Before the experiments, in order to maintain the animals in the same feeding and venom production status, the snakes were fed with two mice each and water was available *ad libitum*. After a period of 30–40 days, when the glands were presumably filled with venom, experiments were begun. The snakes were not fed during this period in order to obtain a greater number of cells at the same stage (quiescent or activated). Venom glands were obtained from snakes unmilked previously and snakes milked 4, 7 or 15 days before they were killed by decapitation, in order to observe any changes occurring during the secretory cycle. During this period between venom extraction and killing the animals, no food was offered to prevent possible stimulation of the venom glands due to loss of venom. For manual venom extraction (Belluomini, 1967), the snakes were anaesthetized using subcutaneous sodium pentobarbital injection (20 mg kg^{-1}, Cristália, Brazil).

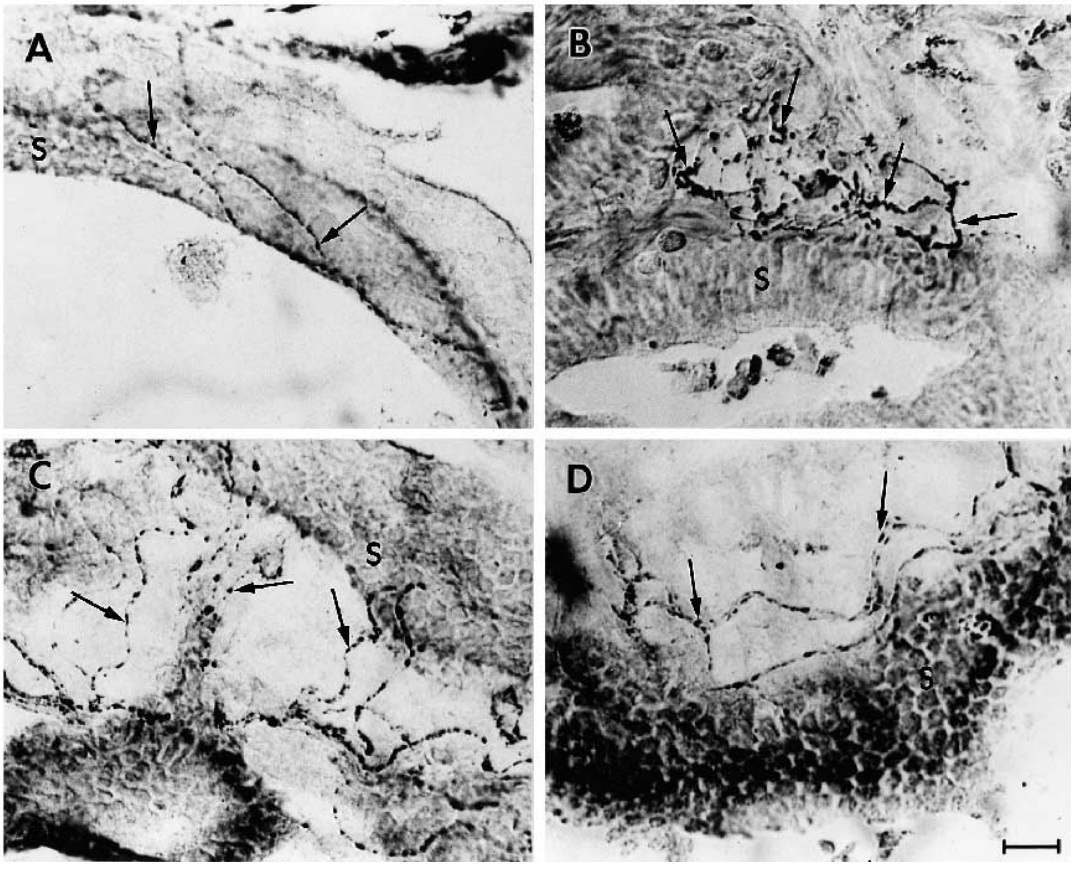

Fig. 2. Sections of *Bothrops jararaca* venom glands in different stages of venom production stained using anti-tyrosine hydroxylase antiserum (arrows). The glands were obtained from unmilked snakes (A), or snakes milked 4 (B), 7 (C) or 15 (D) days previously. S, secretory cells. Scale bar, 20 µm.

Table 1. *Concentration of noradrenaline in* Bothrops jararaca *venom glands obtained from unmilked snakes (day 0) and snakes milked 4, 7 or 15 days previously*

Time after milking (days)	Noradrenaline content (ng mg^{-1} wet tissue)
0	2.99±0.67
4	3.07±0.93
7	2.23±0.83
15	3.02±0.80

Values represent means ± S.E.M., N=5 snakes per day.

Immunohistochemistry

Snakes, unmilked (N=3) or milked 4 (N=3), 7 (N=2) or 15 (N=2) days before they were killed, were anaesthetized using sodium pentobarbital (30 mg kg^{-1} subcutaneously) perfused through the ventricle with cold phosphate-buffered saline (PBS) in order to remove the blood, and then with cold 2 % paraformaldehyde in PBS at a rate of 11 ml min^{-1}. After the perfusion, the venom glands were removed, dissected and kept in the fixative solution for 6 h at 4 °C. They were then transferred to a 30 % sucrose solution in PBS and maintained at 4 °C. Longitudinal sections of 20 µm were obtained using a cryostat, mounted onto gelatin-coated slides and processed for tyrosine hydroxylase (TH) immunohistochemistry using the avidin–biotin technique as described by Britto *et al.* (1988). The primary (omitted in controls) and secondary antibodies used were anti-TH rabbit antiserum (Eugenetech) and biotin-labelled goat anti-rabbit IgG (Jackson Labs), respectively, and biotin–avidin complex (Vector Labs).

High-performance liquid chromatography (HPLC)

Catecholamine determination in venom glands obtained from snakes unmilked and milked 4, 7 or 15 days before they were killed (N=5 snakes per group) was performed by HPLC coupled with electrochemical detection based on the method described by Naffah-Mazzacoratti *et al.* (1992). The glands were homogenized in 0.1 mol l^{-1} perchloric acid containing 0.02 % Na_2EDTA, 0.02 % $Na_2S_5O_5$ and a known concentration of dihydroxybenzylamine (DHBA) (Sigma Chemical Co., St Louis, MO, USA) as an internal standard (30 µl mg^{-1} wet tissue), using a Polytron homogenizer (Brinkmann). The homogenates were frozen overnight and centrifuged at 11 000 ***g*** at 4 °C for 50 min; the supernatants were stored at −70 °C. The extracts were filtered using 0.22 µm filters before being injected (20 µl) into the HPLC apparatus (Shimadzu Corporation).

Light and electron microscopy

Unmilked or milked (4 or 15 days before they were killed) venom glands obtained from control (N=5) or reserpine-treated snakes (N=6, 20 mg kg^{-1}, subcutaneously, 24 h before milking, and 5 mg kg^{-1}, subcutaneously, daily for 15 days as a maintenance dose) (Sigma Chemical Co., St Louis, MO, USA) were prepared as described by Carneiro *et al.* (1991). Semi-thin sections (0.5 µm) were analysed using a light microscope, and ultrathin sections (70 nm) were analysed using a transmission electron microscope (Jeol JEM 1010). Isoprenaline or phenylephrine (100 mg kg^{-1}, subcutaneously, Sigma Chemical Co., St Louis, MO) were also administered (from the fourth to the fifteenth day after milking) to reserpine-treated snakes (N=2 and N=3, respectively), and their effects on venom gland morphology were analysed.

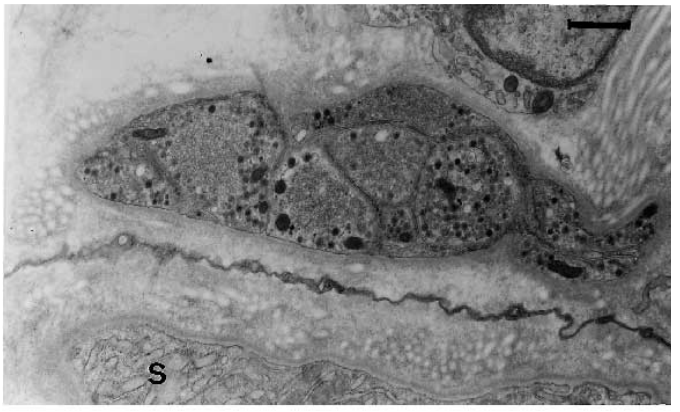

Fig. 3. Electron micrograph showing the nerve terminal in the intertubular conjunctive space near the basal region of the secretory cell in a control unmilked *Bothrops jararaca* venom gland. Note the presence of small vesicles and dense-cored vesicles. S, secretory cell. Scale bar, 1 µm.

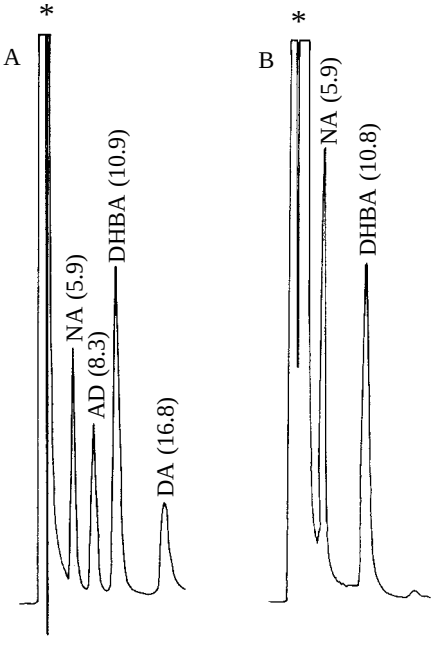

Fig. 4. Typical chromatograms of catecholamines. (A) Catecholamine standards; (B) extract of venom gland obtained from *Bothrops jararaca* milked 4 days previously. NA, noradrenaline; AD, adrenaline; DHBA, 3,4-dihydroxybenzylamine (internal standard); DA, dopamine; *, perchloric acid. Elution time (in min) is given in parentheses.

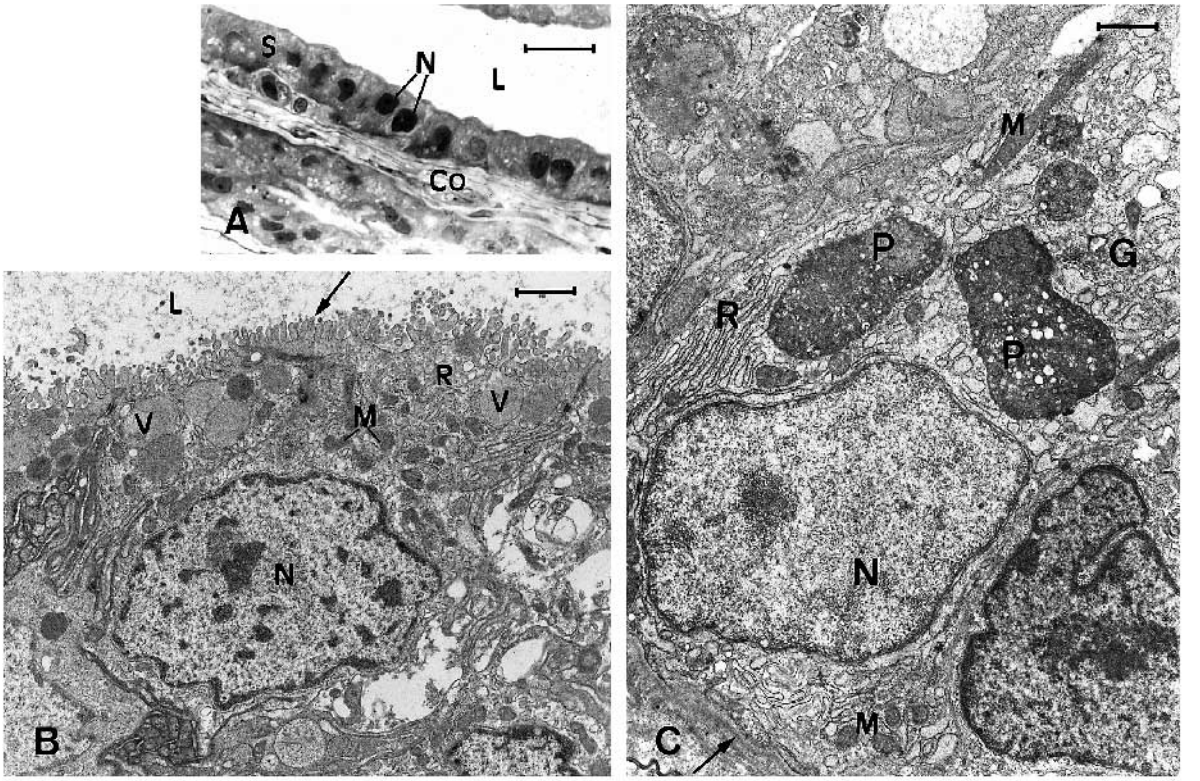

Fig. 5. (A) Light-microscopic view of a semi-thin sections of *Bothrops jararaca* venom gland obtained from an unmilked snake. Note the cuboidal shape of the secretory cells. Scale bar, 30 μm. (B,C) Electron micrographs of ultrathin sections of venom gland secretory cells obtained from unmilked (B) and unmilked reserpine-treated (C) snakes. Note in B that the apical membrane exhibits numerous microvilli (arrow) and that the secretory cell has a very prominent nucleus, poorly developed rough endoplasmic reticulum, electron-dense mitochondria and secretory vesicles. Note in C that the secretory cell has large electron-dense phagosomes near the nucleus and a rough endoplasmic reticulum as stacked narrow cisternae near the nucleus or as more dilated cisternae near the Golgi apparatus. The arrow in C indicates the basal membrane. Co, conjunctive intertubular space; G, Golgi apparatus; L, lumen; M, mitochondria; N, nucleus; P, phagosome; R, rough endoplasmic reticulum; S, secretory epithelium; V, secretory vesicle. Scale bars, 1 μm.

Statistical analysis

Noradrenaline concentrations are expressed as means ± S.E.M. and data are compared using one-way analysis of variance (ANOVA).

Results

Detection of catecholaminergic innervation

The presence of catecholaminergic innervation in the venom glands was verified by detection of tyrosine hydroxylase (TH), the rate-limiting enzyme in the pathway for the synthesis of catecholamines. Positive immunoreaction to TH was observed near the secretory cells of *Bothrops jararaca* venom glands (Fig. 1), and the intensity of the immunoreaction varied according to the stage of venom production of the glands (Fig. 2). The highest immunoreactivity was found in venom glands collected on the fourth day after milking, when compared with unmilked snakes or snakes milked on days 7 or 15.

Nerve terminals located in the control (unmilked) venom gland contained larger numbers of small vesicles (40–50 nm) than of dense-cored vesicles (70 nm) (Fig. 3).

The only catecholamine detected by HPLC was noradrenaline (Fig. 4), confirming the presence of noradrenergic nerve terminals. Adrenaline and dopamine were not detected. The noradrenaline content measured in venom glands obtained from snakes at different stages of venom production did not vary significantly ($P>0.05$) during the protein-secretion cycle (Table 1).

Functional studies

In order to investigate the physiological relevance of the noradrenergic innervation on venom synthesis and secretion, sympathetic activity was blocked with reserpine. No venom could be collected after reserpine treatment. Morphological alterations in the secretory cells of the venom glands were investigated using light and electron microscopy. In accordance with the morphological changes observed in other viperid snakes, the secretory cells of unmilked snake venom gland were flattened and cuboidal in shape (Fig. 5A), and the RER cisternae were narrow even in unmilked reserpine-treated snake venom gland (Fig. 5B,C). After milking, the secretory cells increased in size and assumed a columnar shape, the RER

intracisternal space expanded, many secretory vesicles appeared near the apical membrane, and the Golgi apparatus became well developed (Figs 6A,B, 7A,B). These morphological changes indicate an increase in the rates of protein synthesis and secretion. In reserpine-treated animals (Figs 5C, 6C,D, 7C,D), despite modifications to their shape (Figs 6C,D, 7C,D), the secretory cells remained in the quiescent stage even when the snakes were milked (Figs 6D, 7D). Instead of the wide RER cisternae observed in untreated milked venom glands, narrow RER cisternae and no electron-dense secretory vesicles were found, indicating little or no venom production. In the 15-day reserpine-treated snakes, larger fused secretory vesicles with a less electron-dense content, localized near the apical membrane, were observed (Figs 7C,D, 8) and the Golgi apparatus was less developed than in control snakes (Fig. 9B).

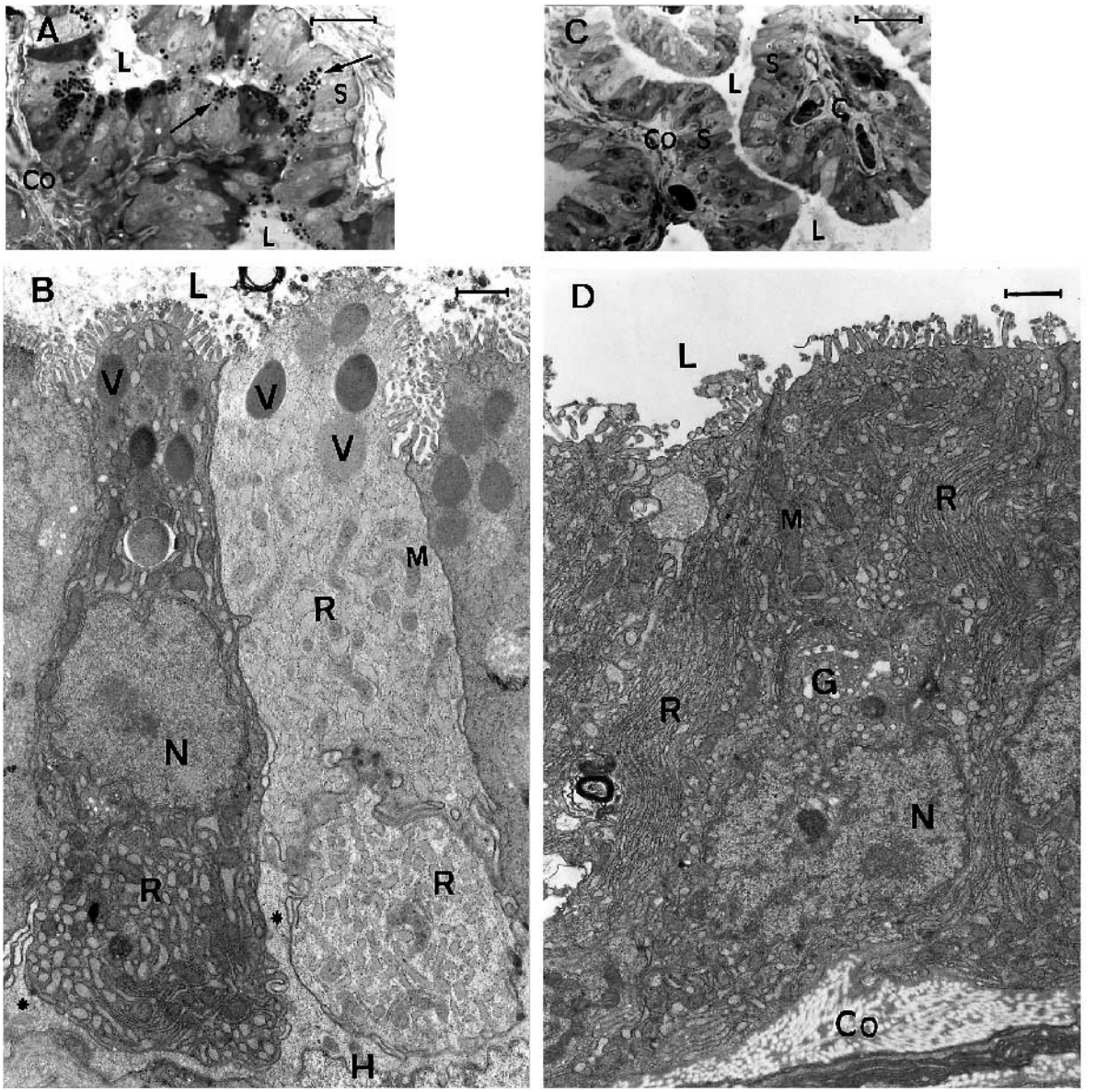

Fig. 6. (A,C) Light microscopic views of semi-thin sections of *Bothrops jararaca* venom gland obtained from a snake milked 4 days previously (A) and a reserpine-treated snake milked 4 days previously (C). Note the columnar shape of the secretory cells in both, but only in A does the secretory epithelium contain numerous apical dense secretory vesicles (arrows). Scale bars, 30 μm. (B,D) Electron micrographs of ultrathin sections of venom gland secretory cells obtained from a snake milked 4 days previously (B) and a reserpine-treated snake milked 4 days previously (D). Note in B that the secretory cell contains irregularly dilated rough endoplasmic reticulum cisternae and electron-dense vesicles in the apical region. In the reserpine-treated snake (D), the majority of the rough endoplasmic reticulum is represented by narrow orderly, stacked cisternae, emergent secretory vesicles are not seen in the supra-nuclear Golgi apparatus and the apical cytoplasm is devoid of dense secretory vesicles. Co, conjunctive intertubular space; G, Golgi apparatus; H, horizontal cell with its extremities indicated by asterisks; L, lumen; M, mitochondria; N, nucleus; R, rough endoplasmic reticulum; S, secretory epithelium; V, secretory vesicle. Scale bars, 1 μm.

After daily subcutaneous administration (from day 4 to day 15 after milking) of phenylephrine (100 mg kg^{-1}) or isoprenaline (100 mg kg^{-1}) to reserpine-treated animals, venom could be collected. However, these α- and β-agonists had different effects on venom gland cell morphology. Both agonists promote RER expansion of cisternae (Fig. 10A,B) similar to that occurring in untreated milked snakes (Fig. 7B). However, only phenylephrine reversed the effect of reserpine on the Golgi apparatus. After phenylephrine treatment, the Golgi apparatus became well developed and emerging secretory vesicles (Fig. 9C), with very similar morphology to those found in control milked venom gland (Fig. 9A), were observed. In contrast, after isoprenaline treatment, the Golgi apparatus remained less well-developed (Fig. 9D), and it appeared that in some secretory cells the RER and apical membrane fused, releasing the RER cisternae contents into the gland lumen (Fig. 11).

Discussion

In the present study, the positive immunoreaction for TH

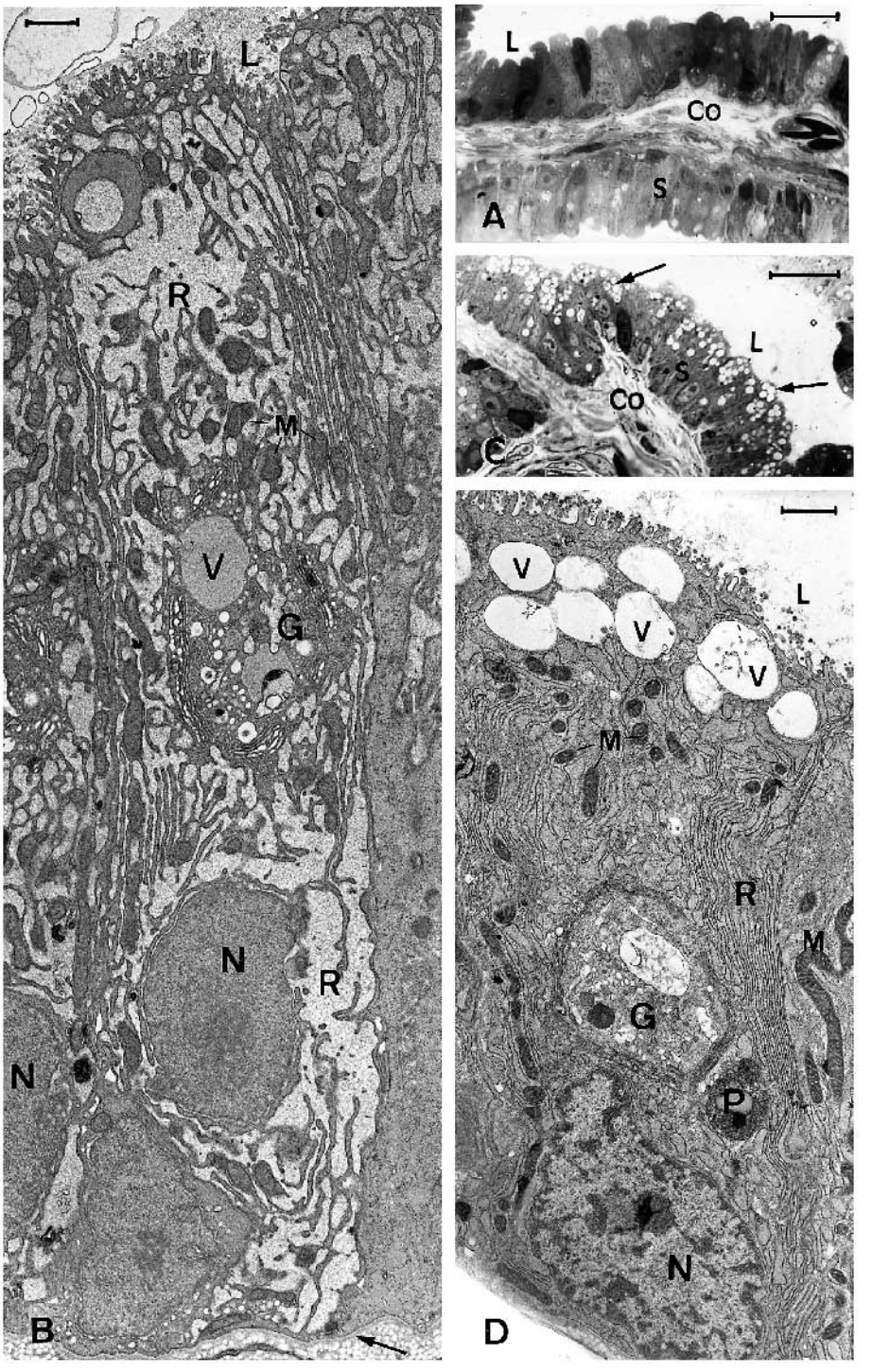

Fig. 7. (A,C) Light-microscopic views of semi-thin sections of *Bothrops jararaca* venom gland obtained from a snake milked 15 days previously (A) and a reserpine-treated snake milked 15 days previously (C). Note the columnar shape of the secretory cells in both figures; only in C does the secretory epithelium contain numerous apical vacuolized vesicles (arrows). Scale bars, 30 μm. Electron micrographs of ultrathin sections of venom gland secretory cells obtained from a snake milked 15 days previously (B) and a reserpine-treated snake milked 15 days previously (D). Note in B that the secretory cell contains irregularly dilated rough endoplasmic reticulum cisternae and the Golgi apparatus area is well developed, with secretory vesicles. In the reserpine-treated snake (D), narrow stacked cisternae of rough endoplasmic reticulum, apical electron-lucent vesicles and less well-developed supra-nuclear Golgi apparatus are seen. Co, conjunctive intertubular space; G, Golgi apparatus; L, lumen; M, mitochondria; N, nucleus; P, phagosome; R, rough endoplasmic reticulum; S, secretory epithelium; V, secretory vesicle. Scale bars, 1 μm.

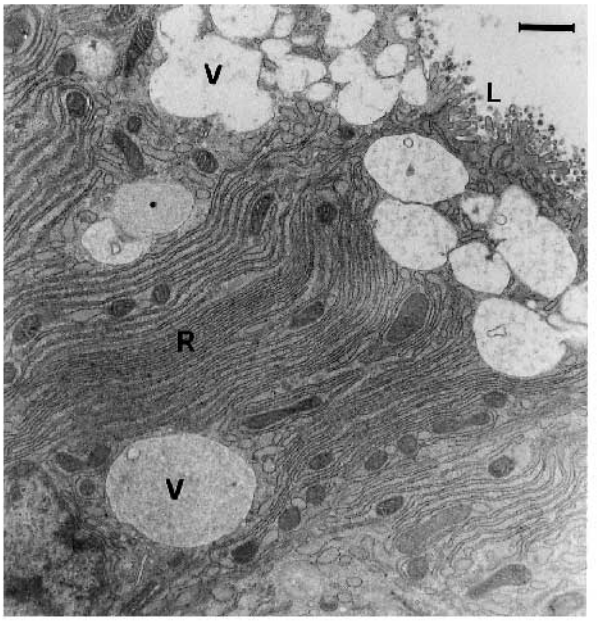

Fig. 8. Fused secretory vesicles in secretory cells of *Bothrops jararaca* venom gland obtained from reserpine-treated snakes milked 15 days previously. Note the rough endoplasmic reticulum with narrow stacked cisternae. L, lumen; R, rough endoplasmic reticulum; V, secretory vesicle. Scale bar, 1 μm

near the secretory cells and the detection of noradrenaline demonstrate the presence of noradrenergic fibres in *Bothrops jararaca* venom glands. Further, the presence of both small and large vesicles, a characteristic of noradrenergic termini (Fillenz, 1990), reinforces the identification of these fibres as noradrenergic. The noradrenaline concentration in the venom gland is higher than in rat and mouse salivary glands (Murai *et al.* 1995). Noradrenaline concentration did not change significantly during the venom production cycle, although the highest positive immunoreaction for TH was found 4 days after milking, when maximal activity of the secretory cells was also observed. This suggests that sympathetic neurotransmission plays a role in the induction of venom production. An increase in noradrenaline turnover may explain the lack of change in noradrenaline concentration during the venom production cycle.

In mammalian salivary glands, the stimulation of β-adrenoceptors is the most important step in the stimulation of protein synthesis (Lillie and Han, 1973; Mehansho and Carlson, 1983; Kim *et al.* 1989; Baum, 1987; Woon *et al.* 1993). To determine whether sympathetic innervation is important for venom production, the effect of pharmacological denervation created by reserpine application on the morphology of venom gland secretory cells was evaluated. Venom could not be collected in reserpine-treated snakes, probably as a result of the impairment of protein synthesis. Morphological analyses of the secretory cells confirmed that venom production and secretion were impaired in reserpine-treated snakes. In untreated snakes, milking led to an expansion of the RER and stimulation of the Golgi apparatus; however, reserpine treatment blocked both

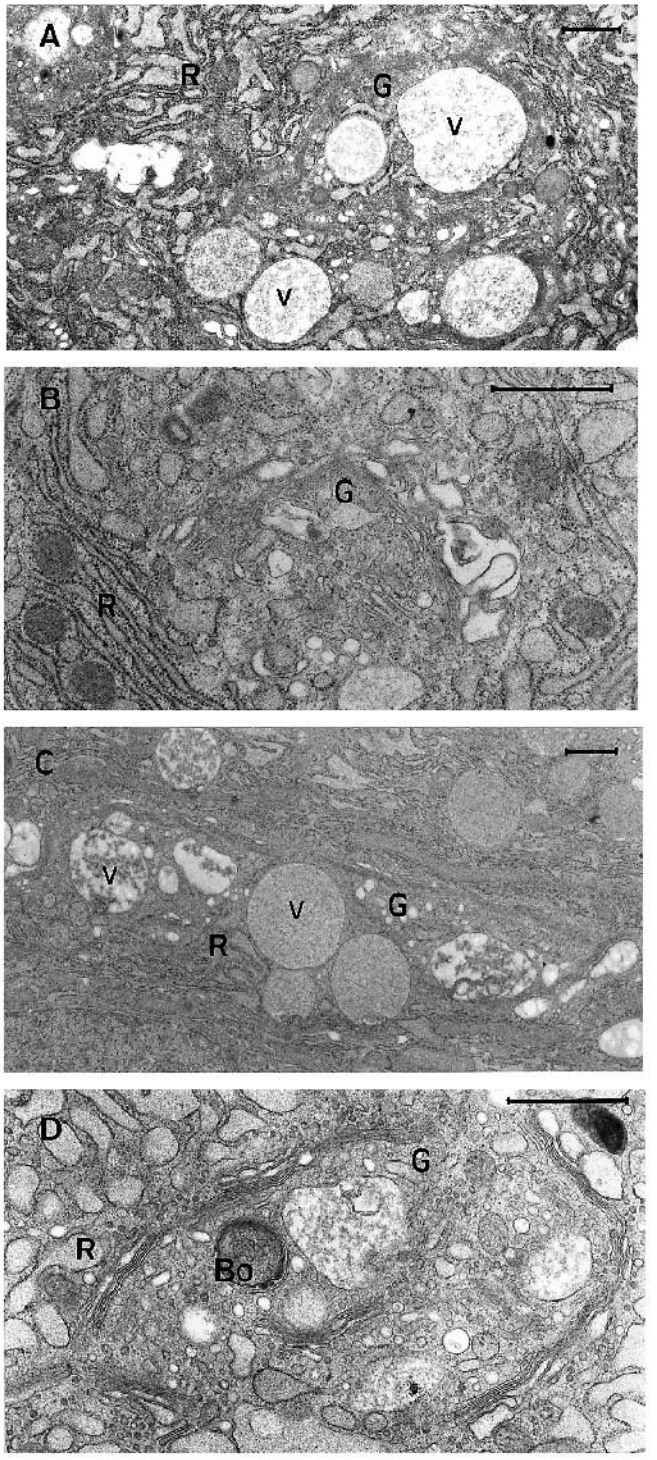

Fig. 9. Golgi apparatus in secretory cells of *Bothrops jararaca* venom gland obtained from (A) a snake milked 4 days previously, (B) a reserpine-treated snake milked 15 days previously, and (C,D) reserpine-treated snakes after administration of phenylephrine (C) or isoprenaline (D). Bo, multivesicular body; G, Golgi apparatus; R, rough endoplasmic reticulum; V, secretory vesicles. Scale bars, 1 μm

responses. In treated animals, the RER cisternae were narrow, the electron-lucent secretory vesicles were larger and some were fused in the apical region of the cytoplasm, and the vesicle

contents were less dense than those in control milked snakes. Similar results were observed in the parotid glands of chronically isoproterenol-treated rats, and this effect was attributed to an impairment of protein synthesis due to receptor desensitization (Vugman and Hand, 1995). Besides impairing protein synthesis, reserpine treatment also affected the Golgi secretory process. Even after milking, the Golgi apparatus remained quiescent, suggesting that protein secretion was inhibited.

To characterize further the role of noradrenergic neurotransmission in venom production, the effects of isoprenaline and phenylephrine on venom glands from reserpine-treated snakes were investigated. After stimulation of either the β- or α-adrenoceptors, it was possible to collect venom, suggesting that both agonists are required for venom production. However, morphological analysis showed that each agonist induced specific changes. In the presence of either isoprenaline or phenylephrine, expansion of RER cisternae in reserpine-treated snakes could be observed, suggesting that stimulation of either β- or α-adrenoceptors induces protein synthesis. However, only phenylephrine promoted the emergence of secretory vesicles from the Golgi apparatus, suggesting that this agonist restores the protein-secretion process. In the isoproterenol-treated snakes, in spite of venom production, the Golgi apparatus was not well developed and it appeared that in some secretory cells the RER and apical membranes had fused, suggesting that the venom extracted from these animals was released directly from the RER to the lumen. Thus, these data suggest that isoprenaline and phenylephrine have similar effects on the RER, but that only phenylephrine restores secretory vesicle formation, confirming the physiological importance of noradrenergic innervation for venom synthesis and secretion.

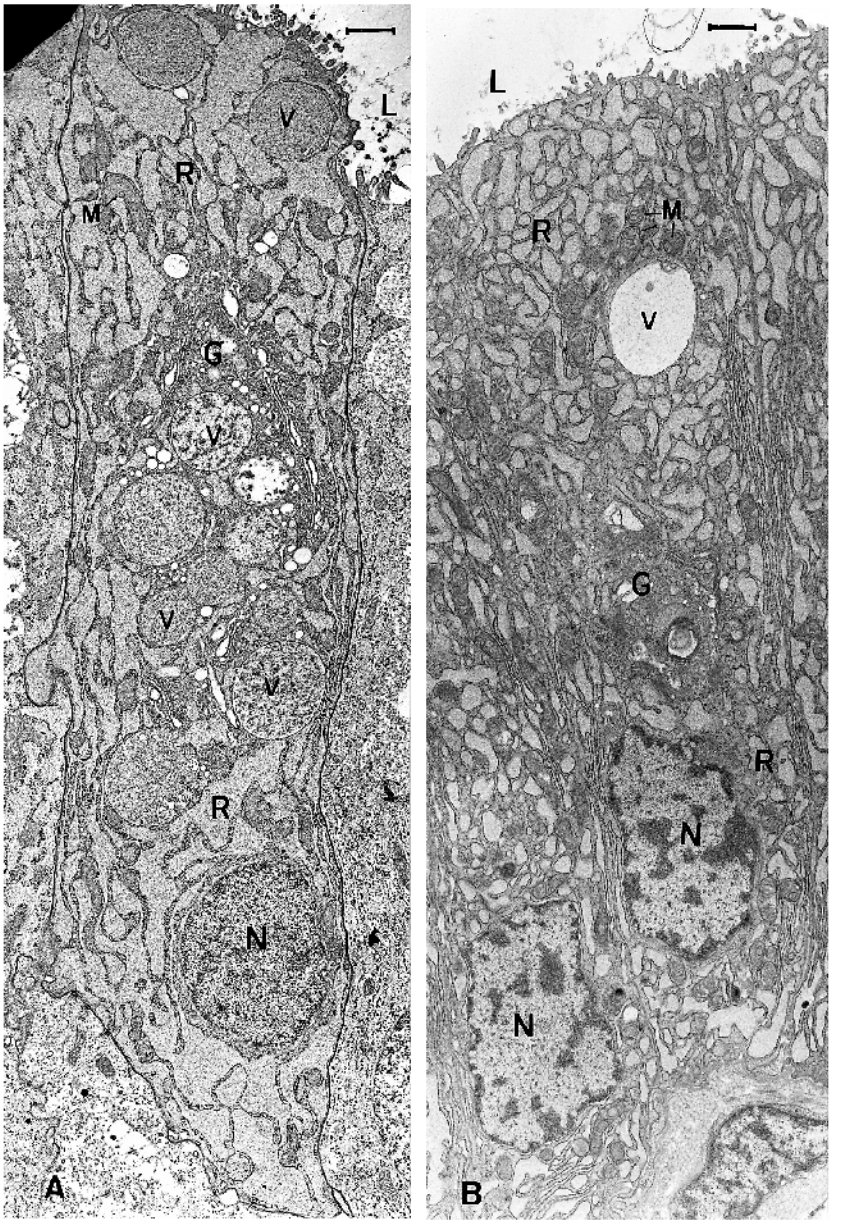

Fig. 10. Effect of phenylephrine (A) or isoprenaline (B) on secretory cells of reserpine-treated *Bothrops jararaca* venom gland. Compare with Fig. 7B,D. (A) A columnar secretory cell containing dilated rough endoplasmic reticulum cisternae and a well-developed supra-nuclear Golgi apparatus with numerous emergent secretory vesicles. (B) Rough endoplasmic reticulum cisternae are moderately dilated, the supra-nuclear Golgi apparatus does not contain emergent secretory vesicles and an atypical large electron-lucent vesicle is observed near the apical region of the cell. G, Golgi apparatus; L, lumen; M, mitochondria; N, nucleus; R, rough endoplasmic reticulum; V, secretory vesicle. Scale bars, 1 μm.

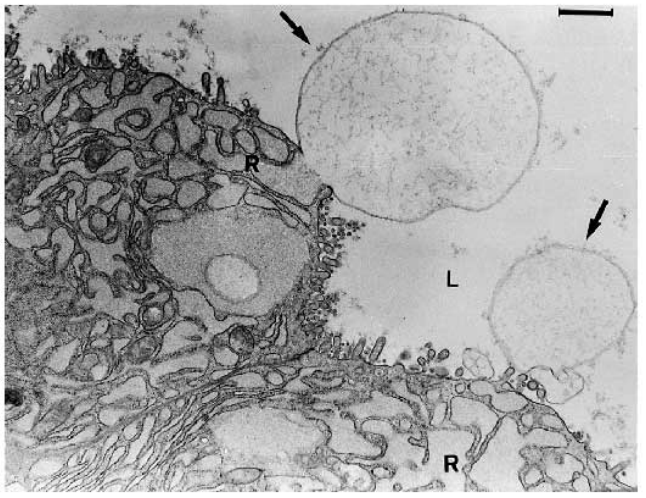

Fig. 11. Atypical secretion of venom in secretory cells of a *Bothrops jararaca* venom gland obtained after administration of isoprenaline in a reserpine-treated snake. Arrows indicate vesicles apparently budding from rough endoplasmic reticulum. L, lumen; R, rough endoplasmic reticulum. Scale bar, 1 μm.

In conclusion, these results provide the first evidence that the sympathetic nervous system plays an important role in the production and secretion of venom in *Bothrops jararaca* (Viperidae) venom glands and that the stimulation of both α- and β-adrenoceptors is involved in protein synthesis, whereas only α-adrenoceptors are involved in protein secretion. An understanding of the importance of noradrenergic stimulation for venom production is fundamental for research on the molecular mechanisms of venom-protein processing and should contribute to the resolution of many long-standing problems in culturing venom secretory cells. Furthermore, it may provide new insights for biotechnological studies aimed at improving snakebite treatment.

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