Effects of ω -conotoxin GVIA on autonomic neuroeffector transmission in various tissues

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1 The effects of ω -conotoxin GVIA (conotoxin), a potent inhibitor of neuronal N-type Ca^{2+} channels, have been examined on responses to stimulation of noradrenergic, cholinergic and non-adrenergic, non-cholinergic (NANC) nerves in a range of isolated tissues to investigate the role of conotoxin-sensitive Ca^{2+} channels in neurotransmission.

2 Contractions elicited by field stimulation of noradrenergic nerves in rat and mouse anococcygeus muscles, rabbit ear artery and rat vas deferens (epididymal portion) were inhibited by conotoxin. Responses to noradrenaline, and to adenosine triphosphate in the vas deferens, were not affected.

3 Positive chronotropic responses to field stimulation of noradrenergic nerves were inhibited by conotoxin in rat and mouse atria, but responses to noradrenaline and tyramine were not affected.

4 The stimulation-induced release of noradrenaline was inhibited by conotoxin in the rabbit ear artery and in rat and mouse atria.

5 Relaxations in response to stimulation of the noradrenergic perivascular mesenteric nerves were reduced or abolished by conotoxin in rat and rabbit jejunum. The response to noradrenaline in rat jejunum was not affected.

6 Contractions elicited by stimulation of cholinergic nerves were inhibited by conotoxin in rat jejunum and mouse ileum (perivascular mesenteric nerves), and in guinea-pig taenia caeci (field stimulation). Responses to acetylcholine in rat jejunum and mouse ileum were not affected.

7 Contractions elicited by stimulation of the cholinergic plus NANC pelvic nerves were inhibited by conotoxin in rabbit colon, and to a lesser extent in guinea-pig colon. The stimulation-induced contraction of the guinea-pig colon was inhibited by conotoxin by a greater proportion in the presence than in the absence of atropine. Responses to acetylcholine were not affected in the rabbit colon but were slightly reduced in the guinea-pig colon.

8 Relaxations in response to field stimulation of NANC nerves were inhibited by conotoxin in guinea-pig taenia caeci and rat gastric fundus strips, and in rat anococcygeus muscle when the tone was raised by guanethidine but not when it was raised by carbachol. The relaxations produced by sodium nitroprusside in the rat gastric fundus and anococcygeus were not affected.

9 Contractions of the rat bladder elicited by stimulation of the peri-urethral nerves, which are NANC- and cholinergically mediated, were relatively insensitive to inhibition by conotoxin. The responses were almost completely abolished by tetrodotoxin.

10 The conotoxin-induced inhibitions of responses to nerve stimulation developed slowly and persisted after removal of conotoxin.

11 The inhibitory effect of conotoxin was inversely proportional to the frequency of stimulation (in several preparations) and to the Ca^{2+} concentration in the bathing solution (in rat vas deferens). These observations suggest that the inhibition by conotoxin of the Ca^{2+} influx required for excitation-secretion coupling in autonomic nerve terminals is not absolute, and can be overcome by repeated stimulation or by raising the Ca^{2+} concentration.

Introduction

Transmitter release from nerve terminals during depolarization produced by invasion of action potentials is thought to depend on the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels (VSCC) of the N-type (Augustine *et al.*, 1987; Miller, 1987). ω -Conotoxin GVIA (referred to as conotoxin henceforth) is a potent inhibitor of neuronal VSCC (Kerr & Yoshikami, 1984; Cruz *et al.*, 1987; 1988; Miller, 1987; Tsien *et al.*, 1988), but does not affect the propagation of action potentials into the terminals of motoneurons (Kerr & Yoshikami, 1984) or sympathetic neurones (Brock *et al.*, 1989).

Conotoxin has been shown to block responses, in autonomically innervated tissues, to stimulation of noradrenergic (Maggi *et al.*, 1988b; Mohy El-Din & Malik, 1988; Brock *et al.*, 1989; Clasbrummel *et al.*, 1989; Keith *et al.*, 1989; McKnight *et al.*, 1989), cholinergic (Lundy & Frew, 1988; Keith *et al.*, 1989) and non-adrenergic, non-cholinergic

(NANC) (Maggi *et al.*, 1988a,b) nerves in a variety of tissues. However, there are some anomalies, since it has been shown that NANC-mediated responses of the rat duodenum (Maggi *et al.*, 1988b) and rat anococcygeus muscle (McKnight *et al.*, 1989) are not sensitive to conotoxin, and the NANC-mediated responses of rat bladder are only slightly inhibited by conotoxin (Maggi *et al.*, 1988b).

It is possible that studies on the inhibition by conotoxin of Ca^{2+} influx through N-type channels might provide new pharmacological and physiological insights into mechanisms of neurotransmission, but further information is needed. Therefore, the aim of the present study was to re-examine some of the apparent anomalies, and to extend the range of tissues used, in an attempt to determine whether or not N-type Ca^{2+} channels are involved in all types of autonomic neurotransmission in all tissues. Preliminary accounts of some of these studies have been communicated to the Australasian Society of Clinical and Experimental Pharmacologists (De Luca & Rand, 1990; De Luca *et al.*, 1990; Li & Rand, 1990a; Reid *et al.*, 1990).

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Methods

Sprague-Dawley rats (200–300 g), New Zealand White rabbits (2–4 kg), random-bred Swiss White mice (20–40 g) and Dunkin-Hartley guinea-pigs (400–600 g) were killed by stunning with a blow to the head and exsanguination, and the required tissues were removed as rapidly as possible.

The physiological salt solution (PSS) used for all the isolated tissues had the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 0.45, NaHCO₃ 25, KH₂PO₄ 1.03, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14.

The reservoirs and organ baths containing PSS were gassed continuously with 5% CO₂ and 95% O₂. The PSS passed through a heat exchanger at 37°C before contact with tissues, and organ baths were maintained at 37°C. Organ baths were silicone coated with Coatasil (Ajax Chemicals, Australia) to prevent adsorption of conotoxin. Preparations were allowed to equilibrate for 30–60 min, with frequent changes of PSS for those mounted in organ baths.

Electrical stimulation was with 0.5–1 ms pulses delivered from Grass S88, S44 or S11 stimulators; supramaximal voltages were used for eliciting responses. Other stimulation parameters (frequency and train length) are described below in Methods and in Results. Extrinsic nerves were threaded through bipolar platinum ring electrodes of the type described by Burn & Rand (1960). Intramural nerves were field stimulated through parallel platinum wires on either side of the tissue.

The activity of longitudinally arranged smooth muscle was measured either isometrically with a strain gauge transducer or isotonically with a Ugo Basile 7006 transducer, as stated below, and was displayed on Rikadenki potentiometric recorders. For isotonic recordings, changes in length were magnified either 5 or 12.5 fold for colon preparations and either 10 or 25 fold for other preparations. Calibrations on records of responses of these tissues have been corrected for amplification.

For all preparations, time control studies were carried out; the functional responses to both nerve stimulation and to exogenously added transmitters remained constant over the time course of the experiments.

Preparation of isolated tissues

Rat and rabbit jejunum Preparations were set up as described by De Luca & Rand (1989). Briefly, segments (rat, about 1.5 cm long; rabbit, 2–3 cm long) were taken from the proximal portion of the jejunum together with the branches of the mesenteric blood vessels supplying the segment and mounted in 20 ml organ baths for isotonic recording (tension: rat, 0.5 g wt; rabbit, 0.7 g wt). The main blood vessel (with the accompanying perivascular nerves) was threaded through bipolar platinum ring electrodes.

Mouse ileum Segments about 1 cm long were taken from the distal portion of the ileum together with the branches of the mesenteric blood vessels supplying the segment. The tension was 0.5 g wt. Other details were as for rat and rabbit jejunum.

Rabbit and guinea-pig colon Segments (3–4 cm in length) of distal colon with the parasympathetic pelvic nerves were dissected out as described by Garry & Gillespie (1954) and set up in 100 ml organ baths for isotonic recording under a tension of 3 g wt for rabbit and 1.5 g wt for guinea-pig colon. The pelvic nerves were threaded through bipolar platinum electrodes.

Guinea-pig taenia caeci Preparations were set up as described by Burnstock *et al.* (1966) in a 5 ml organ bath for isometric recording and field stimulation under a tension of 1 g wt.

Rat gastric fundus Strips of the gastric fundus (approximately 15 mm long and 3 mm wide) were set up as described by Vane (1957) in an 8 ml organ bath for isotonic recording under a tension of 1 g wt and field stimulation. The PSS contained atropine (3 µM) and guanethidine (5 µM) to block cholinergic and noradrenergic involvement, and 5-hydroxytryptamine (5 µM) to raise the tone.

Rat and mouse anococcygeus muscles Anococcygeus muscles were set up, as described by Li *et al.* (1988) for rat and by Gibson & Wedmore (1981) for mouse, in 5 ml organ baths for isometric recording and field stimulation (tension: rat, 1 g wt; mouse, 0.3 g wt).

Rat vas deferens Segments about 1 cm long of the epididymal end of vasa deferentia were set up, as described by McCulloch *et al.* (1985), in a 4 ml organ bath for isometric recording with a resting tension of 1 g wt and field stimulation.

Rat urinary bladder Preparations were set up, as described by Hukovic *et al.* (1965), in a 30 ml organ bath for isometric recording under a tension of 1 g wt. The right urethra with the accompanying nerves was threaded through bipolar platinum electrodes.

Rat and mouse atria Atria were set up in 5 ml organ baths for isometric measurement of spontaneous contractions and field stimulation under resting tensions of 1 g wt for rat and 0.25 g wt for mouse atria, as described by Wong-Dusting *et al.* (1989). The force and rate (derived from a tachometer coupler) of contractions were displayed on a Grass polygraph. Atropine (1 µM) was present in the PSS to block responses to cholinergic nerve stimulation.

Rabbit ear artery Segments of artery were set up for perfusion and superfusion at 4 ml min⁻¹ under a resting tension of 0.5 g wt, as described by Wong-Dusting & Rand (1988). The perivascular nerves were stimulated through bipolar platinum electrodes. Increases in perfusion pressure, measured with a strain gauge manometer, were used as indices of vasoconstrictor responses.

Labelling of noradrenergic transmitter stores with [³H]-noradrenaline and release of radioactivity

Rat and mouse atria and rabbit ear arteries were incubated in (–)-[7,8-³H]-noradrenaline (atria: 0.27 µM, 4 µCi ml⁻¹ for 20 min; arteries: 0.67 µM, 10 µCi ml⁻¹ for 60 min) to label transmitter stores. The stimulation-induced release of radioactivity was used as an index of the release of transmitter noradrenaline as described by Wong-Dusting *et al.* (1989).

Drugs

The drugs used and their sources were: acetylcholine perchlorate (BDH); adenosine triphosphate disodium salt (ATP, Sigma); atropine sulphate (Sigma); carbachol (carbamylcholine chloride, Sigma); ω-conotoxin GVIA (conotoxin, Peninsula); guanethidine sulphate (Ciba); 5-hydroxytryptamine creatinine sulphate complex (Sigma); noradrenaline bitartrate (Sigma); (–)-[7,8-³H]-noradrenaline (specific activity 15 Ci mmol⁻¹, Amersham); phentolamine mesylate (Ciba); sodium nitroprusside (Sigma); tetrodotoxin (Sigma); tyramine hydrochloride (Sigma).

Statistical analysis

Quantitative data are expressed as means and s.e.mean. Differences between means were assessed by Student's *t* test (two-tailed), or by analysis of variance (ANOVA) followed by planned comparisons, as indicated. Analysis by ANOVA was carried out with the software package CSS (Statsoft). Values

of $P < 0.05$ were taken to indicate statistically significant differences.

Results

Gastrointestinal neuroeffector preparations

Rabbit jejunum Stimulation of the mesenteric perivascular nerves with 30 s trains of pulses at 2–20 Hz produced graded inhibitions of pendulum movements and the higher frequencies also caused relaxation, as shown for stimulation at 4, 8 and 16 Hz in Figure 1. Once the frequency-response relationship had been constructed, repeated responses to stimulation producing 50–70% inhibition of pendulum movements were elicited at 3 min intervals until their reproducibility was established. Conotoxin (50 nM) produced a gradual reduction of these responses, and its effect reached a stable level after 15–20 min exposure. After 30 min exposure to conotoxin, the frequency-response relationship was determined again. Responses to stimulation at 2 and 4 Hz were virtually abolished and those at 8 Hz were significantly reduced to $33 \pm 4\%$ ($n = 3$) of the control value ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison) but those at 16 and 20 Hz were not affected (Figure 1).

Rat jejunum The nature of the response to stimulation of the mesenteric perivascular nerves depended on the frequency, as described by De Luca & Rand (1989). Graded contractions were elicited at 0.5, 1 and 2 Hz; there were usually contractions at 5 Hz and relaxations with inhibition of pendulum movements were usually produced at 10 and 20 Hz. Contractions and relaxations elicited in separate preparations by stimulation for 30 s at 5 Hz and 10 Hz, respectively, were greatly attenuated after exposure to 50 nM conotoxin for 15 min, but the corresponding responses to acetylcholine and noradrenaline were not decreased (Figure 2). The residual responses to stimulation were not abolished even when the concentration of conotoxin was increased to 200 nM, but were abolished by tetrodotoxin (1 μ M).

Mouse ileum Stimulation of the mesenteric perivascular nerves at 2–20 Hz elicited frequency-dependent contractions (Figure 3). Acetylcholine (1–10 nM) produced contractions in the same range as those produced by nerve stimulation. Atro-

Figure 1 Responses to stimulation of perivascular mesenteric nerves with 30 s trains at 4, 8 and 16 Hz before and 30 min after exposure to 50 nM conotoxin (CTX) in segments of rabbit jejunum. Isotonic recordings were amplified by 25 fold; the vertical scale has been corrected for the amplification factor. Similar results were obtained in 3 separate experiments.

Figure 2 Effect of conotoxin (50 nM) on responses of segments of rat jejunum to stimulation of perivascular mesenteric nerves and to agonists. Contractions were elicited by stimulation (St, 5 Hz, 30 s) and by acetylcholine (ACh, 100 nM for 30 s) before (a) and 15 min after exposure to conotoxin (b). Relaxations were elicited by stimulation (St, 10 Hz, 30 s) and by noradrenaline (NA, 10 μ M for 30 s) before (c) and 15 min after exposure to conotoxin (d). Isotonic recordings were amplified by 25 fold; the vertical scale has been corrected for the amplification factor. The dashed lines project from the ends of event markers for stimulation to the records. Similar observations were obtained in 2 or 3 preparations.

pine (0.1 μ M) abolished responses to acetylcholine and greatly reduced the responses to stimulation, indicating that they were predominantly cholinergically mediated; the small residual response to stimulation was abolished by tetrodotoxin (1 μ M). After the frequency-response relationship had been established, repeated responses to stimulation at 4 Hz were elicited at 3 min intervals, and in the presence of conotoxin (10 nM) these were gradually reduced and blockade was complete after 30 min. When the frequency-response relationship was again determined after exposure to conotoxin for 45 min, responses to stimulation at 8 Hz or less were abolished and those to 16 and 20 Hz were greatly attenuated (Figure 3).

Figure 3 Contractions of a segment of mouse ileum elicited by stimulation of the perivascular mesenteric nerves at 2–20 Hz for 30 s periods before and 45 min after exposure to conotoxin (CTX, 10 nM). Isotonic recordings were amplified by 25 fold; the vertical scale has been corrected for the amplification factor.

With a higher concentration of conotoxin (50 nM), responses to stimulation at 4 Hz were abolished more rapidly and responses to 16 and 20 Hz were barely discernible.

Rabbit colon Graded contractions were elicited by stimulation of the pelvic nerves at 1, 3 and 10 Hz for 30 s periods, and the contractions produced by 0.3–3 μ M acetylcholine fell within the range of the responses to stimulation. Exposure to conotoxin (20 nM) resulted in reductions of responses to stimulation that developed more rapidly and were more complete at 1 Hz than at 10 Hz (Figure 4). In these experiments, acetylcholine (3 μ M) produced an initial contraction of 9.5 ± 2.6 mm ($n = 4$). After exposure to conotoxin for 80 min, the responses to acetylcholine were not significantly affected, being $94.3 \pm 2.9\%$ of those obtained before conotoxin.

Guinea-pig colon Graded contractions were elicited by stimulation of the pelvic nerves at 1, 3 and 10 Hz for 30 s periods. The guinea-pig colon was more sensitive to acetylcholine than the rabbit colon, and contractions produced by 0.1–0.3 μ M acetylcholine fell within the range of the responses to stimulation. Exposure to conotoxin gradually reduced responses to stimulation until they stabilized after 15–30 min to about 75% of control at a concentration of 10 nM, and to about 40% of control at 30 and 100 nM. In contrast to the findings in the rabbit colon, there were no differences in the rate of development or the extent of the inhibitory effect of conotoxin at the different frequencies of stimulation, and responses to acetylcholine in the guinea-pig colon were reduced to 75–80% of control by 30 and 100 nM conotoxin. The addition of atropine (0.1 μ M) after conotoxin had produced its full effect resulted in a reduction of about 50% in the remaining response. The residual response in the presence of conotoxin and atropine was abolished by tetrodotoxin (1 μ M).

The effect of conotoxin was tested on the non-cholinergic component of the response to stimulation in the presence of atropine (0.1 μ M), which abolished responses to acetylcholine and reduced responses to stimulation to about 30% of the initial value; in addition, an initial phase of relaxation was revealed. Exposure to conotoxin (30 nM) reduced the initial relaxation and the contraction (Figure 5), and produced a greater maximum reduction in contractile responses to stimulation (at 10 Hz for 30 s) in the presence of atropine (87% reduction) than in the absence of atropine (64% reduction).

Figure 4 Effect of conotoxin (20 nM) on contractions of rabbit colon elicited by stimulation of the extrinsic pelvic nerves ($\circ = 1$ Hz, $\square = 3$ Hz, $\triangle = 10$ Hz) for 30 s periods. Responses are expressed as percentages of stable control responses before exposure to conotoxin. The horizontal axis is time of exposure to conotoxin. Symbols are means and vertical lines s.e.means ($n = 4$), which were sometimes smaller than the size of the symbol. The control isotonic contractions (corrected for amplification) were: 1 Hz, 7.8 ± 2.5 mm; 3 Hz, 11.3 ± 2.4 mm; 10 Hz, 12.3 ± 1.6 mm. All responses in the presence of conotoxin were significantly different from the appropriate control responses ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison), except that at 3 Hz for 5 min. The effect of conotoxin was significantly dependent on both time and frequency ($P < 0.05$, two-way ANOVA with repeated measures).

Figure 5 Effect of conotoxin (CTX, 30 nM) on responses of a segment of guinea-pig colon to stimulation (St, 10 Hz, 30 s) of the pelvic nerves in the presence of atropine (Atr, 0.1 μ M), which abolished the response to acetylcholine (ACh, 0.1 μ M) and reduced the stimulation-induced contraction, revealing an initial relaxation. Numbers above the records are times (in min) of exposure to conotoxin. Isotonic recordings were amplified by 5 fold; the vertical calibration has been corrected for amplification.

Guinea-pig taenia caeci Field stimulation (2 Hz, 5 s) elicited cholinergically-mediated contractions. These were gradually reduced by conotoxin (20 nM for 30 min) to $15.3 \pm 1.9\%$ ($n = 4$) of control responses.

NANC-mediated relaxations in response to field stimulation (0.5 Hz, 5 s) were revealed in the presence of atropine (0.3 μ M) and guanethidine (5 μ M) (Burnstock *et al.*, 1966). These responses were gradually reduced to $52 \pm 4.5\%$ ($n = 4$) and $20 \pm 3.9\%$ ($n = 4$) of control responses after exposure to conotoxin in concentrations of 20 nM and 100 nM, respectively, for 30 min.

Rat gastric fundus NANC-mediated relaxations were elicited by field stimulation with 60 s trains at 1, 2 and 5 Hz in the presence of atropine (3 μ M) and guanethidine (5 μ M) to block cholinergic and noradrenergic involvement, and 5-hydroxytryptamine (5 μ M) to raise the tone. Exposure to conotoxin (50 nM) for 30 min resulted in loss of the response to stimulation at 1 Hz; responses to stimulation at 2 and 5 Hz were inhibited more slowly to $21.6 \pm 3.5\%$ and $44.2 \pm 6.6\%$ ($n = 3$) of control responses, respectively. Relaxations elicited by sodium nitroprusside (50 nM) were not significantly affected, being $92.3 \pm 6.2\%$ ($n = 3$) of initial responses after 30 min exposure to conotoxin (50 nM), and in time-control experiments without conotoxin they were $98.7 \pm 4.5\%$ ($n = 4$) of initial responses.

Pelvic neuroeffector preparations

Rat anococcygeus muscle Frequency-dependent noradrenergically-mediated contractions were elicited by field stimulation (0.5–10 Hz for 10 s periods). Exposure to conotoxin (20 nM) for 15 min abolished responses to stimulation at 0.5 and 1 Hz, but had a lesser inhibitory effect as the frequency of stimulation was increased (Figure 6a). Responses to noradrenaline (0.1 μ M) were not significantly affected.

Relaxations in response to field stimulation of NANC nerves were revealed by blocking noradrenergically-mediated contractions and raising the tone either with guanethidine (10–20 μ M), as first described by Gillespie (1972), or with phenolamine (3 μ M) plus carbachol (30 μ M), as described by McKnight *et al.* (1989). In the presence of guanethidine, exposure to conotoxin (20 nM) for 15 min almost abolished relaxations elicited by 0.5 Hz and greatly reduced those elicited by 1 and 2 Hz stimulation, but responses to stimulation at 5 and 10 Hz were not greatly affected (Figure 7). In the presence of phenolamine plus carbachol, the frequency-response curve for relaxations was to the right of that obtained in the presence of guanethidine, and these responses were not significantly reduced by 20 nM conotoxin (Figure 7). Relaxations elicited by sodium nitroprusside (10 nM) were not significantly affected by conotoxin: after 30 min exposure to 20 nM conotoxin and in time-control experiments without conotoxin the relaxations

Figure 6 Contractions elicited by field stimulation (0.5–10 Hz for 10 s periods) in rat (a, $n = 5$) and mouse (b, $n = 4$) anococcygeus muscles, before (○) and after (●) 15 min exposure to conotoxin (20 nM). Responses are expressed as percentages of those elicited initially by stimulation at 10 Hz. Symbols are means and vertical lines show s.e.means, which were smaller than the size of the symbol in some cases. An asterisk indicates a significant difference between responses obtained before and after exposure to conotoxin ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison).

were $103 \pm 4.9\%$ ($n = 4$) and $109 \pm 7.2\%$ ($n = 5$), respectively, of those obtained initially.

Mouse anococcygeus muscle The effects of exposure to conotoxin (20 nM) for 15 min on noradrenergically-mediated contractions elicited by field stimulation (0.5–10 Hz for 10 s periods) are shown in Figure 6b. Responses to stimulation at all frequencies were significantly reduced.

Rat urinary bladder Frequency-dependent contractions were elicited by stimulation (0.5–50 Hz for 10 s periods) of the peri-urethral nerves. The effects of conotoxin in one experiment are illustrated in Figure 8a. After exposure to conotoxin (20 or 100 nM) for 30 min, responses to stimulation were reduced to an increasing extent by increasing the frequency from 0.5 to 2 Hz, but were reduced to a decreasing extent as the frequency

Figure 7 Relaxations of rat anococcygeus muscles elicited by field stimulation (0.5–10 Hz for 10 s periods) in the presence of guanethidine (10–20 μM; ○, ●; $n = 5$) or in the presence of phentolamine plus carbachol (3 μM + 30 μM; △, ▲; $n = 4$), before (○, △) and after (●, ▲) 15 min exposure to conotoxin (20 nM). Responses are expressed as percentages of those elicited initially by stimulation at 10 Hz. Symbols are means and vertical bars show s.e.means, which were smaller than the size of the symbol in some cases. An asterisk indicates a significant difference between responses in the presence of guanethidine obtained before and after exposure to conotoxin ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison). Conotoxin had no significant effect on responses obtained in the presence of phentolamine plus carbachol.

Figure 8 Effect of conotoxin on contractions of rat bladder elicited by stimulation of the peri-urethral nerves (10 s trains at 0.5–50 Hz). (a) Stimulation-induced contractions before and after 30 min exposure to conotoxin (CTX, 20 nM). (b) Inhibition of responses 30 min after exposure to 20 (○) or 100 (□) nM conotoxin or 1 μM tetrodotoxin (△). Inhibitions are expressed as percentage reductions from initial responses at each frequency of stimulation. An asterisk indicates a significant difference between responses obtained in the presence and absence of conotoxin ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison). Symbols represent means and vertical lines show s.e.means ($n = 3$ in each case), which were smaller than the size of the symbol in some cases.

was further increased to 50 Hz (Figure 8b). The maximum reduction, which occurred at 2 Hz, was about 50% of the control response. The responses were abolished or reduced to about 10% of the control values by tetrodotoxin (1 μM) (Figure 8b).

Rat vas deferens Contractions of epididymal portions were elicited by field stimulation using a wide range of frequencies and train lengths. Responses to single pulses of stimulation were gradually reduced and abolished after 20 min of exposure to conotoxin (50 nM). Stimulation at frequencies of 0.5–2 Hz elicited responses in which individual contractions were clearly discernible; the first two were abolished and remaining ones were reduced by conotoxin (Figure 9a,b,c). At higher frequencies of stimulation, smooth contractions were elicited (e.g. Figure 9d,e) and these were reduced by conotoxin to extents that were inversely proportional to the train length and frequency of stimulation. Responses to noradrenaline (10 μM) and ATP (3 mM) were not significantly affected by conotoxin.

Changes in the Ca^{2+} concentration of the PSS The effects of conotoxin (50 nM) were investigated in the presence of a raised (5 mM) and lowered (0.625 mM) Ca^{2+} concentration on responses to stimulation that were, respectively, markedly reduced (5 pulses at 5 Hz) and only slightly reduced (10 pulses at 10 Hz) by conotoxin in a PSS with a Ca^{2+} concentration of 2.5 mM. The findings are summarized in Table 1. Compared to its effects in a PSS containing 2.5 mM Ca^{2+} , conotoxin produced a smaller reduction of the control responses when the Ca^{2+} concentration was 5 mM, and a greater reduction of the

Figure 9 Responses of an epididymal portion of rat vas deferens to field stimulation (a, 5 pulses at 0.5 Hz; b, 11 pulses at 1 Hz; c, 21 pulses at 2 Hz; d, 20 pulses at 5 Hz; e, 10 pulses at 10 Hz) before (left hand records) and after (right hand records) 120 min exposure to conotoxin (CTX, 50 nM). Similar observations were obtained in 3 experiments.

control responses when the Ca^{2+} concentration was 0.625 mM. Conotoxin had no effect on responses to noradrenaline (10 μM) or ATP (3 mM) in either high or low Ca^{2+} PSS.

Cardiovascular preparations

Rabbit ear artery Vasoconstrictor responses to stimulation of the perivascular sympathetic nerves with 10 s trains at 0.5, 1 and 2 Hz were gradually reduced in the presence of conotoxin (10, 20 and 30 nM), but vasoconstrictor responses to nor-

Figure 10 Effect of conotoxin on vasoconstrictor responses elicited by stimulation of the perivascular nerves in the rabbit ear artery. (a) Responses to noradrenaline (NA, 20 ng) and to 10 s trains of pulses at 0.5, 1 and 2 Hz, respectively, before and 60 min after exposure to conotoxin (CTX, 30 nM). (b) Mean results from experiments of the type illustrated in (a) after 60 min exposure to 10 (O), 20 (□) or 30 (Δ) nM conotoxin. Responses are expressed as percentages of those to each frequency of stimulation before exposure to conotoxin; the mean ($\pm$ s.e.mean) control increases in perfusion pressure for stimulation at 0.5, 1 and 2 Hz were 28.4 ± 7.1 , 46.6 ± 11.3 and 60.8 ± 13.4 mmHg, respectively. Symbols are means and vertical lines show s.e.means ($n = 4$ for each concentration of conotoxin). All responses in the presence of conotoxin were significantly different from the appropriate control responses ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison). The effect of conotoxin was significantly dependent on both frequency and concentration of conotoxin ($P < 0.05$, two-way ANOVA with repeated measures).

adrenaline were not reduced (Figure 10). The rate of development of the inhibitory effect of conotoxin on responses to nerve stimulation was concentration-dependent, the effect being fully developed in less than 30 min with 30 nM but only after 45 min with 10 nM conotoxin. The maximal fully developed reductions of responses to nerve stimulation were concentration-dependent and inversely proportional to the frequency of stimulation (Figure 10b). The inhibitory effect persisted for at least 30 min after the conotoxin was removed.

In separate experiments, the stimulation-induced (2 Hz for 10 s) efflux of radioactivity from arteries previously incubated in [^3H]-noradrenaline was reduced to about 60% of the corresponding control value by exposure for 30 min to 40 nM conotoxin (Table 2). This reduction in noradrenaline release was in reasonable accordance with the reduction in the vasoconstrictor response to stimulation. The reduction in the stimulation-induced release of radioactivity not only persisted after the removal of conotoxin but was greater, being about 40% of the corresponding control value (Table 2).

Table 1 Effects of changes in the Ca^{2+} concentration of the PSS on the inhibition by conotoxin (50 nM) of field stimulation-induced contractions of rat vasa deferentia

Stimulation Pulses	Hz	Extracellular [Ca^{2+}] (mM)	Response (% control)	n
5	5	2.5	17.6 ± 8.8	3
5	5	5	$59.8 \pm 8.0^*$	3
10	10	2.5	67.3 ± 22.4	3
10	10	0.625	8.0 ± 7.0	4

Responses are expressed as percentages of the peak tension produced by stimulation before exposure to conotoxin. Values shown are means $\pm$ s.e.means; n = number of experiments. An asterisk indicates a significant difference from the appropriate control with 2.5 mM Ca^{2+} ($P < 0.05$, unpaired t test).

Table 2 Stimulation-induced effluxes of radioactivity from tissues in which transmitter stores of noradrenaline had been labelled with [^3H]-noradrenaline

CTX	n	First period (S_1 , d.p.m.)	Second period (% of S_1)	Third period (% of S_1)
Rabbit ear artery				
0	15	3002 $\pm$ 312	86.0 $\pm$ 6.4	73.1 $\pm$ 7.3
40 nM	6	2892 $\pm$ 396	50.0 $\pm$ 4.4*	29.5 $\pm$ 4.1*
Rat atria				
0	4	3965 $\pm$ 282	81.2 $\pm$ 8.3	76.2 $\pm$ 6.6
10 nM	4	4784 $\pm$ 409	14.3 $\pm$ 2.0*	22.8 $\pm$ 1.1*
Mouse atria				
0	4	16452 $\pm$ 2341	100.4 $\pm$ 3.6	
10 nM	4	16787 $\pm$ 1780	36.7 $\pm$ 2.6*	

In the rabbit ear artery and rat atria, three periods of stimulation at 2 Hz for 10 s were given. In mouse atria, two periods of stimulation at 5 Hz for 60 s were given. Stimulation periods were 45 min apart. Conotoxin (CTX) was added 30 min before the second period and removed immediately after the last collection of PSS for that period. Effluxes in the second and third periods of stimulation are expressed as percentages of effluxes in the first period of stimulation (S_1). Values are mean $\pm$ s.e.mean; n is the number of experiments. An asterisk indicates a significant difference between values obtained from experiments carried out in the presence and absence of conotoxin ($P < 0.05$, unpaired t test).

Mouse and rat atria Conotoxin (10 nM) attenuated the increases in atrial rate caused by field stimulation (0.4 to 8 Hz for 5 s) of the intramural sympathetic nerves (Figure 11). The responses were decreased to a greater extent with the lower than with the higher frequencies of stimulation, and the inhibitory effect persisted for at least 30 min after conotoxin had been removed. The decreases in responses to sympathetic

nerve stimulation produced by conotoxin were not greater if the exposure time was increased from 30 to 60 min. Conotoxin (10 nM) had no effect on the increases in mouse or rat atrial rates caused by 0.1 μM noradrenaline or 3 μM tyramine (Table 3).

In atria incubated in [^3H]-noradrenaline, conotoxin (10 nM) reduced the stimulation-induced efflux of radioactivity, and the effect in rat atria was shown to persist for 30 min after the removal of conotoxin (Table 2). In the same experiments, the stimulation-induced positive chronotropic responses to sympathetic nerve stimulation were also reduced to approximately the same (rat) or to a lesser (mouse) extent than the effluxes of radioactivity; the increases in atrial rate in the second period of stimulation expressed as percentages of the increase in the first period were 101.0 $\pm$ 6.8% (mouse, $n = 3$) and 101.4 $\pm$ 1.4% (rat, $n = 4$) in control experiments and 68.5 $\pm$ 11.1% (mouse, $n = 4$) and 37.3 $\pm$ 4.5% (rat, $n = 4$) in the presence of 10 nM conotoxin.

Figure 11 Increases in rate of beating produced by field stimulation (0.4, 1, 2, 4 and 8 Hz for 5 s periods, corresponding to 2, 5, 10, 20 and 40 pulses, respectively) in mouse (a) and rat (b) atria before (○) and after (●) exposure to 10 nM conotoxin for 30 min, and 30 min after the removal of conotoxin from mouse atria (□). Symbols are means and vertical lines show s.e.means ($n = 4$). There was a significant difference between responses obtained before and after exposure to conotoxin for all frequencies of stimulation except 0.4 Hz (two-way ANOVA with repeated measures followed by planned comparisons). The inhibitory effect of conotoxin was significantly dependent on frequency of stimulation ($P < 0.05$, one-way ANOVA with repeated measures). In 2 of the 4 experiments with rat atria, the inhibitory effect was shown to persist unchanged for 30 min after removal of conotoxin (data not illustrated).

Discussion

The results show that conotoxin inhibits responses to stimulation of noradrenergic, cholinergic and NANC nerves, but there are some differences in the sensitivity to conotoxin between tissues and between species. However, no clear pattern emerges from the findings of differential sensitivity to conotoxin on responses mediated by different types of transmitters or on transmitters evoking stimulating as opposed to inhibitory responses of effector tissues.

Effects of conotoxin on noradrenergic transmission

Conotoxin inhibited contractions elicited by field stimulation of noradrenergic nerves in epididymal portions of the rat vas deferens, which are predominantly mediated by noradrenaline (McGrath, 1978; Brown *et al.*, 1983). Maggi *et al.* (1988a) have

Table 3 Increases in atrial rate (beats min^{-1}) produced by 0.1 μM noradrenaline and 3 μM tyramine in rat and mouse isolated atria before and after 30 min exposure to conotoxin (CTX, 10 nM)

	Noradrenaline		n	Tyramine		n
	Before CTX	After CTX		Before CTX	After CTX	
Rat	108 $\pm$ 15	103 $\pm$ 8	4	91 $\pm$ 13	98 $\pm$ 9	4
Mouse	151 $\pm$ 8	158 $\pm$ 8	4	100 $\pm$ 12	103 $\pm$ 13	4

Values are means $\pm$ s.e.means; n = number of experiments.

previously shown that conotoxin also inhibits responses to field stimulation in the prostatic portion of the rat vas deferens, where ATP is the predominant transmitter (Sneddon & Westfall, 1984). Noradrenergic nerve-mediated contractions of the rat anococcygeus muscle were inhibited by conotoxin, confirming the finding of McKnight *et al.* (1989). However, responses in the mouse anococcygeus were appreciably less affected than those in the rat, suggesting a species difference in the sensitivity to conotoxin. Vasoconstrictor responses to noradrenergic nerve stimulation were inhibited by conotoxin in the rabbit ear artery. It has been previously demonstrated that conotoxin inhibits stimulation-induced vasoconstriction in the rat perfused kidney (Mohy El-Din & Malik, 1988) and rat tail artery (Clasbrummel *et al.*, 1989). Relaxations of smooth muscle elicited by noradrenergic nerve stimulation were inhibited by conotoxin in the rat and rabbit jejunum. Positive chronotropic responses to noradrenergic nerve stimulation were inhibited by conotoxin in rat and mouse atria.

The stimulation-induced release of noradrenaline was inhibited by conotoxin (10 nM) in rat and mouse atria by about 80% and 60%, respectively, but the reduction was less in the rabbit ear artery (40%), even though the concentration of conotoxin (40 nM) was higher. These differences in sensitivity to conotoxin do not appear to be related to differences in the stimulation parameters (see Table 2), and presumably reflect differences in the susceptibility to conotoxin between species and tissues. It has been demonstrated previously that conotoxin inhibits electrical stimulation-induced release of noradrenaline from rat perfused kidneys (Mohy El-Din & Malik, 1988), tail artery (Clasbrummel *et al.*, 1989) and anococcygeus muscle (McKnight *et al.*, 1989).

Effects of conotoxin on cholinergic transmission

Conotoxin inhibited contractions elicited by field stimulation of cholinergic nerves of the guinea-pig taenia caeci and cholinergic nerve-mediated components of contractions elicited by extrinsic nerve stimulation in rat jejunum, mouse ileum, and rabbit and guinea-pig colon. The inhibition by conotoxin was less complete in the guinea-pig colon than in the other preparations. It is possible that cholinergic transmission in the guinea-pig colon is relatively conotoxin-resistant. An alternative explanation is that in the guinea-pig colon there may be interactions at postjunctional sites of transmitters with contracting and relaxing actions and at prejunctional sites with transneuronal modulation of transmitter release, and this complexity obscures the effect of conotoxin.

Inhibition of responses to field stimulation of cholinergic nerves has been shown previously in field-stimulated guinea-pig ileum preparations (Lundy & Frew, 1988; Keith *et al.*, 1989).

Effects of conotoxin on NANC transmission

Inhibition by conotoxin of contractions elicited by stimulation of NANC nerves was observed in rabbit and guinea-pig distal colon, and to some extent in the rat bladder. Inhibition of NANC-mediated relaxations by conotoxin was demonstrated in the guinea-pig taenia caeci, and in the rat anococcygeus muscle and gastric fundus.

In the rat anococcygeus, there is evidence indicating that nitric oxide (NO) is involved in the transmission process with low frequencies of stimulation (Li & Rand, 1989b). NANC-mediated relaxations of the rat anococcygeus muscle elicited by low frequencies of field stimulation were inhibited by conotoxin, when they were revealed by blocking the response to stimulation of the noradrenergic nerves and raising the tone with guanethidine. However, when NANC-mediated relaxations were revealed by blocking the response to noradrenergic nerve stimulation with phentolamine and raising the tone with carbachol, they were not significantly affected by conotoxin, as demonstrated previously by McKnight *et al.*

(1989). The NANC relaxations revealed in the presence of guanethidine by stimulation at 0.5 Hz are approximately matched by those revealed in the presence of carbachol by stimulation at 5 Hz. The loss of reactivity to low frequencies of stimulation in the presence of carbachol is due to the inhibition of release of the NANC transmitter by activation of prejunctional muscarinic receptors (Li & Rand, 1989a). The higher frequencies of stimulation necessary to produce relaxations in the presence of carbachol may explain the lack of effect of conotoxin (see below). Alternatively, the prejunctional muscarinic receptors may be coupled to N-type Ca^{2+} channels in such a way that the sensitivity of the channels to conotoxin is diminished when the muscarinic receptors are activated.

Contractions of the rat bladder elicited by stimulation of the peri-urethral nerves, which are NANC- and cholinergically mediated (Maggi *et al.*, 1988b), were relatively insensitive to conotoxin, as was previously found by Maggi *et al.* (1988b) who elicited contractions by field stimulation of the intramural nerves. Maggi *et al.* (1988b) showed that responses to stimulation at 0.1 Hz were only reduced by about 25% by 10–300 nM conotoxin, whereas contractions of the guinea-pig bladder were reduced by about 70% by 10 nM to 1 μM conotoxin. In contrast to findings with other tissues in which responses elicited by low frequencies of stimulation were reduced by a greater proportion than those elicited by higher frequencies, the inhibition of stimulation-induced contractions of the rat bladder increased with increasing frequency of stimulation from 0.1 to 5 Hz (where it was about 50%) and only then decreased as the frequency increased, as was also found by Maggi *et al.* (1988b). It is possible that transmitter release from nerve terminals in the bladder does not only involve Ca^{2+} influx through conotoxin-sensitive channels. An alternative suggestion is that the stimulation-induced contractions of the bladder are the result of the actions of transmitters having contractile and relaxant actions, and that the various transmitters also engage in automodulation and transneuronal modulation of transmitter release, such that reductions in their release do not greatly alter the response. Testing of this suggestion must await further elucidation of the transmitters involved and the development of selective blocking drugs. An example of the complexity of the innervation of the bladder has been demonstrated by Andersson *et al.* (1989) in the dog, in which hypogastric nerve stimulation produced a small contraction followed by relaxation and pelvic nerve stimulation produced a biphasic contraction, the first phase being NANC-mediated and the second being cholinergic.

In the rabbit colon, the contractions elicited by stimulation of the pelvic nerve supply were reduced by about 20% by 0.1 μM atropine, which completely abolished the response to acetylcholine, and were reduced by about 50% by 300 μM hexamethonium (Garry & Gillespie, 1955; Thaina *et al.*, 1989). Thus, a considerable proportion of the response is due to stimulation of nerve fibres that do not conform to the expectations for pre- or postganglionic cholinergic parasympathetic neurones. Whatever the nature of the NANC transmitter mediating part of the contractile response, the process of transmitter release is conotoxin-sensitive, since stimulation-induced contractions were almost completely abolished.

In the guinea-pig colon, the contractions elicited by stimulation of the pelvic nerves were reduced by 30–50% by 0.1 μM atropine, which completely abolished the response to acetylcholine, and were reduced by about 80% by 300 μM hexamethonium (Thaina *et al.*, 1989). Hence, the component that cannot be attributed to stimulation of pre- or postganglionic cholinergic parasympathetic neurones is less than in the rabbit colon. However, in contrast to the findings with the rabbit colon, conotoxin was relatively ineffective in the guinea-pig colon, in that it only reduced stimulation-induced responses by about 50%. After the cholinergic component of the response had been eliminated with atropine, conotoxin produced a greater reduction in the response. Thus, the conotoxin

insensitivity cannot be assigned to the NANC-mediated component.

Relative sensitivities to conotoxin of different types of transmission in the same organ

In some cases, conotoxin appeared to be equally effective in inhibiting different transmission processes in the same organ. For instance, in the rat jejunum, cholinergically-mediated contractions and noradrenergically-mediated relaxations were equally inhibited, even though the latter were elicited with a higher frequency of stimulation. Furthermore, in the rat anococcygeus there was little difference between the sensitivity to conotoxin of the NANC-mediated relaxations (in the presence of guanethidine) and the noradrenergically-mediated contractions.

However, in other cases there was differential sensitivity to conotoxin between different transmission processes, for example, in rat gastric fundus strips and in guinea-pig taenia caeci.

In the rat gastric fundus, it has been suggested that stimulation-induced relaxations are largely mediated by vasoactive intestinal peptide (VIP) when prolonged trains (5 min) of stimulation at 5 Hz are used (Lefebvre, 1986), but are predominantly mediated by nitric oxide (NO) when lower frequencies or shorter train lengths of stimulation are used (Li & Rand, 1990b). Conotoxin abolished the response to stimulation at 1 Hz and slowed the rate of development of the responses to stimulation at 2 and 5 Hz, suggesting that it was more effective in inhibiting release of NO than VIP. This would be in accord with the findings that VIP release from enteric nerves does not depend on extracellular Ca^{2+} and is not affected by the inorganic Ca^{2+} channel blocker Cd^{2+} (Belai *et al.*, 1987). However, the higher frequency and longer duration of stimulation necessary for producing the VIP-mediated component of the relaxations may be responsible for its relative insensitivity to conotoxin (see below).

In guinea-pig taenia caeci, conotoxin reduced cholinergically-mediated contractions to a greater extent than NANC-mediated relaxations, even though the latter were elicited at a lower frequency of stimulation. Thus, the Ca^{2+} channels linked to neurotransmitter release in the NANC terminals in guinea-pig taenia caeci may not be identical to those in the cholinergic terminals.

Neuronal selectivity of conotoxin

The selectivity of conotoxin for nerve terminals has been demonstrated by the findings that responses of effector tissues to putative transmitters and other agonists are not impaired when stimulation-induced responses are greatly reduced or abolished. Thus, conotoxin did not affect responses of the rabbit ear artery and rat and mouse atria to noradrenaline, of rat jejunum to acetylcholine or noradrenaline, of rabbit colon to acetylcholine, and of rat gastric fundus and anococcygeus muscle to nitroprusside. However, responses of the guinea-pig colon to acetylcholine were reduced by conotoxin. A possible explanation is that acetylcholine may exert part of its action by exciting neurones in the myenteric plexus. It has previously been shown that conotoxin did not affect responses of various tissues to putative transmitters when responses to nerve stimulation were greatly reduced or abolished (Lundy & Frew, 1988; Maggi *et al.*, 1988a,b; Mohy El-Din & Malik, 1988; Clasbrummel *et al.*, 1989; McKnight *et al.*, 1989).

The lack of effect of conotoxin on the response to tyramine in rat and mouse atria demonstrates that its prejunctional inhibitory action is confined to Ca^{2+} -dependent transmitter release.

It has been found that high concentrations of conotoxin (0.3–10 μM) produced contractions of rat gastric fundus and uterus, and enhanced responses of the uterus to acetylcholine and KCl (Ichida *et al.*, 1988). We did not observe any such

effects with the lower concentrations of conotoxin used in any of the tissues we studied.

General properties of conotoxin in inhibiting transmitter release

We found that the inhibitory effect of conotoxin on neurotransmission was slow to develop in all the preparations used, as has been noted previously for NANC transmission in the rat heart (Maggi *et al.*, 1988a) and prostatic segments of the rat vas deferens (Maggi *et al.*, 1988b), and for noradrenergic transmission in rat tail artery (Clasbrummel *et al.*, 1989) and rat kidney (Mohy El-Din & Malik, 1988).

It has also been shown in the present study that the rate of development of the inhibitory effect of conotoxin is proportional to its concentration for cholinergic transmission in the mouse ileum and noradrenergic transmission in the rabbit ear artery, as previously demonstrated for NANC transmission in the guinea-pig bladder (Maggi *et al.*, 1988b), for release of tachykinins from sensory terminals in guinea-pig bronchial smooth muscle (Maggi *et al.*, 1988a) and for noradrenergic transmission in the rat tail artery (Clasbrummel *et al.*, 1989). However, this relationship did not occur in the guinea-pig colon.

The extent of the inhibitory effect of conotoxin was concentration-dependent in the field-stimulated guinea-pig ileum (Lundy & Frew, 1988) and rat tail artery (Clasbrummel *et al.*, 1989), and we observed this in the rabbit ear artery. However, the residual responses in the presence of conotoxin in rat jejunum, mouse ileum, guinea-pig colon and rat bladder were not affected by increasing the concentration of conotoxin, although they were tetrodotoxin-sensitive.

A further determinant of the rate of development and of the extent of the inhibitory action of conotoxin is the frequency of stimulation. We have not found a previous statement to this effect in the literature, but we consider it to be an important feature of the action of conotoxin. In some preparations, the inhibitory effect of conotoxin was virtually complete at low frequencies of stimulation but became less as the frequency was increased and in some was virtually absent at a sufficiently high frequency of stimulation. Such effects were observed for noradrenergic transmission in the rabbit jejunum, rabbit ear artery, rat and mouse anococcygeus muscles, rat and mouse atria, and rat vas deferens, for cholinergic transmission in the mouse ileum, and for cholinergic plus NANC transmission in the rabbit colon, and for NANC transmission in the rat anococcygeus muscle. We suggest that the most likely explanation for the frequency-dependent effect of conotoxin is that it reduces the influx of Ca^{2+} into nerve terminals, that follows invasion of the terminals with a single action potential, to below the threshold for activation of excitation-secretion coupling, but the intracellular Ca^{2+} concentration can accumulate to the point that it does trigger excitation-secretion coupling when there are successive invasions of the terminals at a sufficiently high frequency. This explanation fits well for the effect of conotoxin in abolishing the first and second contractions of the rat vas deferens elicited by a train of pulses at low frequencies of stimulation and the gradual increase in size of subsequent contractions (Figure 9). A decrease in the inhibitory effect of conotoxin as the number of pulses in a train was increased was noted by Clasbrummel *et al.* (1989).

Support for the view that conotoxin impedes but does not abolish completely the influx of Ca^{2+} into nerve terminals comes from the finding that its inhibitory effect is inversely proportional to the Ca^{2+} concentration, as demonstrated previously for cholinergic transmission in the frog neuromuscular junction (Kerr & Yoshikami, 1984; Sano *et al.*, 1987) and noradrenergic transmission in the rat tail artery (Clasbrummel *et al.*, 1989), and as found in the present study, for noradrenergic transmission in the rat vas deferens. In frog dorsal root ganglion cells, the depression of the Ca^{2+} current by conotoxin was

partly overcome by increasing the extracellular Ca^{2+} concentration (Oyama *et al.*, 1987).

Another feature of the action of conotoxin is the persistence of its effect after its removal. We observed this in the various preparations we used, and studied it particularly in rat and mouse atria, in which the effect persisted unchanged for at least 30 min, and in the rabbit ear artery, in which the inhibition became even greater after removal of conotoxin. The persistence of the effect of conotoxin has been noted by many other investigators in a variety of systems; for example, inhibition of acetylcholine release from the myenteric plexus of guinea-pig ileum (Lundy & Frew, 1988) and inhibition of neuroeffector transmission in the rat tail artery (Clasbrummel *et al.*, 1989) and vas deferens (Brock *et al.*, 1989).

Conclusions

Conotoxin acts on nerve terminals to inhibit autonomic noradrenergic, cholinergic and NANC neuroeffector transmission in a wide range of tissues, although some are relatively more sensitive than others. The inhibitory effects of conotoxin are inversely proportional to the frequency of stimulation and to the Ca^{2+} concentration of the PSS. These observations

suggest that the inhibition by conotoxin of the Ca^{2+} influx required for excitation-secretion coupling in autonomic nerve terminals is not absolute, and can be overcome by repetitive stimulation or by raising the Ca^{2+} concentration. Possible explanations for the relative insensitivity of transmission to conotoxin in some neuroeffector systems may be because (i) they do not require Ca^{2+} influx for excitation-secretion coupling, (ii) Ca^{2+} influx is not through N-type channels, (iii) different types of nerve terminals may be endowed with sub-types of N-type channels having different sensitivities to conotoxin, or (iv) Ca^{2+} influx has already been partly inhibited by autoinhibitory or transneuronal modulation, thereby obscuring the effect of conotoxin.

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