ω -Conotoxin GVIA is a potent inhibitor of sympathetic neurogenic responses in rat small mesenteric arteries

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1 We have investigated the effects of the N-type calcium channel blocker, ω -conotoxin GVIA, on contractile responses to nerve stimulation, noradrenaline and KCl in rat small mesenteric arteries. In separate experiments, single and summated excitatory junctional potentials (e.j.ps) evoked by nerve stimulation were recorded with an intracellular electrode in the absence and presence of ω -conotoxin.

2 Electrical field stimulation of intramural sympathetic nerves (30 V; 0.25 ms pulse width; 3 s train length; 4–24 Hz) caused frequency-dependent contractions. Cumulative concentration-response curves for the contractions induced by noradrenaline and KCl were constructed in the same preparations. Stimulation at 0.2 Hz and 10 Hz induced respectively single e.j.ps without contractions and summated e.j.ps associated with a contractile response.

3 ω -Conotoxin (0.1 to 3 nM) inhibited markedly and in a concentration-dependent manner both the contractions and e.j.ps to electrical field stimulation. The concentration-response curves to exogenous noradrenaline and KCl remained unaffected.

4 The time-course for the effects of ω -conotoxin (0.3 to 3 nM) indicated a slow onset of action with at least one hour to achieve an equilibrium.

5 The experiments indicate that ω -conotoxin acts prejunctionally to inhibit sympathetic neurotransmission in rat small arteries presumably by inhibition of noradrenaline release. We suggest that ω -conotoxin could be a useful tool to study the control of vascular tone through the autonomic nervous system.

Introduction

The depolarization-induced release of noradrenaline from nerve terminals is triggered by a presynaptic influx of extracellular calcium ions through voltage-operated channels (Reichardt & Kelly, 1983; Smith & Augustine, 1988). Recent electrophysiological studies have shown that voltage-operated calcium channels (VOCCs) can be subdivided in L-, N- and T-type on the basis of differences in unitary barium conductance and gating properties (Nowycky *et al.*, 1985; Miller, 1987). These three types of channel also differ in their tissue distribution and pharmacological properties (Tsien *et al.*, 1988). Thus, N-type VOCCs have been demonstrated exclusively in neuronal preparations (Nowycky *et al.*, 1985; McCleskey *et al.*, 1987). Patch-clamp studies have clearly demonstrated that ω -conotoxin GVIA (ω -CTX), a 27 amino acid peptide isolated from the marine snail *Conus geographus*, is a selective and potent inhibitor of L- and N-type calcium channels in dorsal ganglion, sensory, sympathetic and hippocampal neurones of vertebrates but not in cardiac, skeletal or smooth muscle cells (McCleskey *et al.*, 1987). ω -CTX inhibited synaptosomal ⁴⁵Ca influx (Reynolds *et al.*, 1986) and neurotransmitter release from synaptosomes (Reynolds *et al.*, 1986), cultured neurones (Hirning *et al.*, 1988) and nerve terminals in the rat and guinea-pig vas deferens (Maggi *et al.*, 1988). In the guinea-pig vas deferens, ω -CTX abolished stimulation-evoked contractions and excitatory junctional potentials (e.j.ps) (Brock *et al.*, 1989).

Surprisingly, even though the sympathetic neuromuscular transmission in arteries is of crucial importance in the maintenance of vascular resistance, no direct information is available to date on the possible modulation by ω -CTX of sympathetic neurotransmitter release from nerve endings in the vasculature. In the present study, we used ω -CTX to explore the role of N-type calcium channels in the release of the sympathetic neurotransmitter in resistance arteries of the rat mesentery. To dissociate prejunctional from postjunctional sites of action, concentration-contractile response curves to

KCl and noradrenaline were generated in the absence and presence of ω -CTX. The action of ω -CTX on e.j.ps evoked by nerve stimulation was also investigated since e.j.ps have been demonstrated to be mediated by ATP rather than noradrenaline in rat mesenteric arteries (Angus *et al.*, 1988).

Methods

Wistar-Kyoto rats (10 to 15 weeks) were killed by CO₂ (Kraus, 1980) and small arteries from the superior mesenteric bed were carefully dissected under a Nikon 102 microscope. Mesenteric vessels were then mounted as ring preparations in an isometric myograph (J.P. Trading, Denmark) as previously described (Angus *et al.*, 1988). During dissection, mounting and the course of experiments, arteries were held in a physiological salt solution (PSS) of the following composition (in mM): NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.17, NaHCO₃ 25, CaCl₂ 2.5, ethylene-diaminetetracetic acid (EDTA) 0.026, glucose 5.5, bubbled with 95% O₂ plus 5% CO₂. The vessels were set up in a 15 ml bath and stretched to an internal circumference equal to 0.9 L₁₀₀ where L₁₀₀ is the internal circumference the vessel would have when relaxed and under a pressure of 100 mmHg (Mulvany & Halpern, 1977). Square wave field stimulation was achieved through 5 μ m thick platinum electrodes contained on the mounting supports of the myograph with a low-output-resistance stimulator (Grass SD9/S88, Quincy, MA, U.S.A.). Membrane potential was measured by conventional intracellular capillary-glass microelectrodes (resistances of about 100 M Ω) filled with 0.5 mM KCl solution. The reference electrodes were similarly prepared and positioned close to the impaling electrode. The output of the force transducer amplifier was recorded on a potentiometric recorder (Model 320, W&W Scientific Instruments, Basel, Switzerland). In electrophysiological experiments, signals of force transducer and electrode amplifiers were recorded simultaneously on a potentiometric recorder and on a dual-beam storage oscilloscope (5113 Tektronix, Beaverton, OR, U.S.A.). After being mounted, the vessels were activated successively with KCl (124 mM), 10 μ M

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noradrenaline in KCl (124 mM) and 10 μ M noradrenaline. Field stimulation (0.25 ms, 30 V) was then applied at 4–24 Hz for 3 s every minute. The field stimulation was repeated after irreversible blockade of autinhibitory prejunctional α_2 -adrenoceptors by benextramine (3 μ M) treatment, added in the presence of prazosin (0.1 μ M) to protect α_1 -adrenoceptors. After 5 min exposure to benextramine and prazosin, the vessels were repeatedly washed with drug-free PSS to remove the reversible postjunctional α_1 -adrenoceptor antagonist prazosin but leaving the prejunctional α_2 -adrenoceptors irreversibly blocked with benextramine (see Angus *et al.*, 1988). Arteries were then equilibrated with desipramine (0.1 μ M) and atropine (1 μ M) to avoid interference from neuronal uptake or cholinergic activity.

In pilot experiments conducted to assess the optimum equilibration period for ω -conotoxin, five 3 s trains of field pulses at 24 Hz were applied at one minute intervals before and 5, 20, 40 and 60 min after addition of ω -conotoxin or its vehicle. In the definitive experiments, two 3 s trains of field pulses at 4, 8, 16 and 24 Hz were applied at one minute intervals before and one hour after incubation with vehicle (H_2O) and ω -conotoxin (0.1, 0.3, 1 or 3 nM). Only one concentration was tested in each preparation. In the same experiments concentration-response curves to noradrenaline (0.1–30 μ M) or to KCl (5–70 mM) were obtained before and one hour after equilibration of the vessel with ω -CTX. In electrophysiological experiments, the tissue bath (15 ml) was perfused at 5 ml min⁻¹ with oxygenated PSS. This continuous perfusion system helped to maintain the electrode impalement. The time to reach the appropriate concentration of a drug in the bath when added to the perfusate was approximately 1 min. Field pulses were applied at either 25 V, 0.25 ms, 0.2 Hz to obtain repetitive e.j.s without measurable contraction or at 10 Hz for 500 ms to achieve e.j.p. summation and a small contraction. Thirty minutes after ω -CTX (3 nM), these two periods of field stimulation were re-applied. Before the application of ω -CTX in all experiments, tetrodotoxin (TTX, 0.1 μ M) was added as a bolus to the bath to confirm that the e.j.s and contractions to field stimulation were neurally mediated.

Drugs

The drugs and suppliers were as follows: ω -conotoxin GVIA (Peninsula Lab.), prazosin hydrochloride (Pfizer), (–)-noradrenaline bitartrate (Sigma), atropine sulphate (Sigma), desipramine hydrochloride (Sigma), benextramine tetrahydrochloride (Sigma), tetrodotoxin (Calbiochem). Solutions of noradrenaline were made fresh daily. Stock solutions of TTX and ω -CTX were stored frozen and diluted daily in distilled water and discarded. Stock solutions of other drugs were kept in the refrigerator.

Statistical analysis

Each contraction concentration-response curve to noradrenaline and KCl was fitted to a logistic equation to determine objectively the EC_{10–90}% values and the maximum contraction (Nakashima *et al.*, 1982). Comparisons of average ($\pm$ 1 s.e.mean) EC₅₀ values and maximum responses were achieved between groups by a non-parametric Kruskal-Wallis test. Significant differences were accepted at the 5% level. For the responses to electrical field stimulation, the average s.e.mean for the responses to the entire range of frequencies within the artery was calculated from a two-way analysis of variance as (error mean square/number of arteries)^{0.5} after the sums of squares between arteries and between frequencies had been subtracted from the total sums of squares. These error bars are placed on the line relating % contraction and frequency at no specific point for each concentration of conotoxin.

Results

Electrical field stimulation at 24 Hz for 3 s caused a contraction in rat mesenteric arteries (150–350 μ m i.d.) of $52.2 \pm 1.7\%$ ($n = 9$) of the maximum contraction to noradrenaline (10 μ M). These contractions were mediated by neural transmission since tetrodotoxin (0.1 μ M) completely abolished the response after 1 min incubation (Figure 1). This effect of tetrodotoxin

Figure 1 Representative tracings from two arteries showing the effects of ω -conotoxin (3 nM) on contractions to electrical field stimulation (30 V, 0.25 ms pulse width, 3 s train every minute, 24 Hz) and to noradrenaline (NA, 10 μ M) in rat small mesenteric arteries. The effect of tetrodotoxin (TTX, 0.1 μ M) is also represented. ω -Conotoxin (ω -CTX) or the corresponding vehicle was incubated with the tissue for one hour. D₁₀₀ is the normalised internal diameter of the vessel and NS = nerve stimulation.

Figure 2 Time-course for the effects of ω -conotoxin on the contractions induced by electrical field stimulation (30 V, 0.25 ms pulse width, 3 s train every min, 24 Hz) in rat small mesenteric arteries. Five consecutive trains of stimulation were evoked before, 5, 20, 40 and 60 min after addition of ω -conotoxin (0.3 (○); 1 (▲); 3 (△) nM) or its vehicle (●). Error bars are ± 1 average s.e.mean ($n = 4-5$) (see Methods). Normalised internal diameter, D_{100} , was $253 \pm 15 \mu\text{m}$ (●), $321 \pm 13 \mu\text{m}$ (○), $249 \pm 22 \mu\text{m}$ (▲) and $267 \pm 18 \mu\text{m}$ (△). Responses are given as fraction of wall tension response to noradrenaline ($10 \mu\text{M}$) (NA_{10} max) at start of experiment which was $2.66 \pm 0.46 \text{ N m}^{-1}$ (●), $3.42 \pm 0.48 \text{ N m}^{-1}$ (○), $2.65 \pm 0.51 \text{ N m}^{-1}$ (▲) and $2.23 \pm 0.27 \text{ N m}^{-1}$ (△).

was rapidly and completely reversed on replacing the bathing fluid with drug-free saline solution. These neurogenic contractions were inhibited by ω -CTX (3 nM) incubated for one hour, as illustrated in Figure 1b. Evidence that ω -CTX, at the most effective concentration on nerve stimulation, did not affect the postjunctional smooth muscle cell reactivity was that the peak response to noradrenaline ($10 \mu\text{M}$) was unaltered after one hour (Figure 1b). ω -Conotoxin (0.3–3 nM) was very slow in onset in reducing the peak response to 3 s electrical stimulation at 24 Hz (Figure 2). After one hour exposure to 0.3, 1 or 3 nM ω -CTX, the contraction to 24 Hz for 3 s was reduced by 38.3 ± 8.9 , 76.3 ± 0.6 and $83.4 \pm 1.8\%$ of the control contraction. The neurogenic responses were reduced by ω -CTX at all frequencies of stimulation and the inhibitory effect was concentration-dependent (Figure 3). At 16 Hz, ω -CTX inhib-

Figure 3 Effects of ω -conotoxin on the contractions induced by electrical field stimulation (30 V, 0.25 ms pulse width, 3 s train every min, 4–24 Hz) in rat small mesenteric arteries. Field stimulation at 4, 8, 16 and 24 Hz was evoked one hour after addition of ω -conotoxin (0.1 (■); 0.3 (○); 1 (▲); 3 (△) nM) or its vehicle (●). Error bars are ± 1 average s.e.mean ($n = 5-6$). Normalised internal diameter, D_{100} , was $304 \pm 20 \mu\text{m}$ (●), $308 \pm 15 \mu\text{m}$ (■), $258 \pm 39 \mu\text{m}$ (○), $296 \pm 32 \mu\text{m}$ (▲) and $298 \pm 22 \mu\text{m}$ (△). Responses given as fraction of wall tension response to noradrenaline ($10 \mu\text{M}$) at start of experiment which was $2.40 \pm 0.38 \text{ N m}^{-1}$ (○), $2.06 \pm 0.30 \text{ N m}^{-1}$ (■), $2.52 \pm 0.29 \text{ N m}^{-1}$ (○), $2.49 \pm 0.28 \text{ N m}^{-1}$ (▲) and $3.00 \pm 0.32 \text{ N m}^{-1}$ (△).

Figure 4 Effects of ω -conotoxin on the contractions induced by KCl (a) and noradrenaline (b). Cumulative concentration-response curves to noradrenaline ($0.1-30 \mu\text{M}$) and KCl ($0-70 \text{ mM}$) were obtained one hour after addition of ω -conotoxin (0.1 (■); 0.3 (○); 1 (▲); 3 (△) nM) or its vehicle (●). Horizontal error bars are s.e.mean ($n = 5-6$) at EC_{50} values. Vessels were the same as in Figure 2. In each case EC_{50} values were not significantly different ($P > 0.05$).

ited the amplitude of contractions by 21.1, 29.4, 76.4 and 87.4% of control values at 0.1, 0.3, 1 and 3 nM respectively. The cumulative concentration-response curves to both KCl ($5-70 \text{ mM}$) and noradrenaline ($0.1-10 \mu\text{M}$) were unaffected by ω -CTX (Figure 4).

Field stimulation at very low frequencies (0.2 Hz) caused e.j.ps of 5–10 mV but no contraction (Figure 5). These e.j.ps

Figure 5 Simultaneous recordings of membrane potential (E_m , upper traces) and wall force (F , lower traces) of a rat small mesenteric artery before and after treatment with tetrodotoxin (a) or ω -conotoxin (b). Stimulation (25 V, 0.25 ms pulse width) was at 0.2 Hz. Tetrodotoxin (TTX) was added as a bolus to the perfused tissue (5 ml min^{-1}) so that the recovery of excitatory junctional potentials (e.j.ps) correspond to the wash-out of TTX (a). ω -Conotoxin (ω -CTX, 3 nM) was infused for 30 min (b). Resting membrane potentials indicated on the figure. The normalised internal diameter of the artery was $310 \mu\text{m}$. Note the 10 mV calibration signal on the E_m records.

Figure 6 Simultaneous recordings of membrane potential (E_m) and wall force (F) of a rat small mesenteric artery before (a) and after treatment with ω -conotoxin (3 nM, ω -CTX) for 30 min (b). Stimulation (25 V, 0.25 ms duration, 500 ms train length) was at 10 Hz. Resting membrane potential indicated on the figure. The normalised internal diameter of the artery was 310 μ m. Note the 10 mV calibration mark.

were totally abolished after 30 min equilibration with ω -CTX (3 nM).

When the frequency of 0.2 Hz was raised to 10 Hz in the absence of ω -CTX the e.j.ps summated and a small contraction resulted (Figure 6). These electrophysiological and mechanical responses were abolished by ω -CTX (Figure 6).

Discussion

The present results clearly indicate that ω -CTX is a potent inhibitor of sympathetic neurotransmission in rat small mesenteric arteries probably by interfering with noradrenaline release. We also demonstrated that ω -CTX was without effect on vascular smooth muscle cells when contractions were induced by α_1 -adrenoceptor activation or by direct smooth muscle depolarization by K^+ .

The small mesenteric arteries of the rat were selected as a preparation with sympathetic innervation on which pre- and post-junctional effects of ω -CTX could be functionally studied. Furthermore, arteries with an internal diameter between 150 and 350 μ m are important in the control of vascular resistance (Mulvany, 1984) and the modulation of their tone by the autonomic nervous system is poorly understood. In these vessels, it has been demonstrated that the contraction to field stimulation is mediated primarily through α -adrenoceptors (Nilsson, 1984; Angus *et al.*, 1988) and it is known that the release of an adrenergic transmitter in response to electrical stimulation of nerve fibres requires the influx of calcium ions into terminals (Reichardt & Kelly, 1983; Smith & Augustine, 1988). However, the mechanism sustaining this process has not yet been established. It is thought that depolarization activates presynaptic VOCCs but these channels are relatively insensitive to classical calcium entry blockers (Beattie *et al.*, 1986; McCleskey *et al.*, 1987; Miller, 1987; Hirning *et al.*, 1988). Nifedipine, diltiazem and verapamil have been shown to inhibit the release of noradrenaline from the rabbit isolated

heart and pulmonary artery, but the concentrations of calcium antagonists required were much higher than those needed to block VOCCs in smooth or cardiac muscle (Starke & Schümann, 1973; Göthert *et al.*, 1979; Zelis *et al.*, 1985). In contrast, the release of neurotransmitter from nerve terminals triggered by presynaptic VOCCs was shown to be inhibited by the 27 amino acid peptide ω -CTX in the rat isolated kidney (Mohy El-Din & Malik, 1988) and in various non-vascular peripheral tissues, such as the vas deferens (Maggi *et al.*, 1988; Brock *et al.*, 1989) and the mesenteric plexus longitudinal muscle (Lundy & Frew, 1988). In the rat isolated urinary bladder and proximal duodenum, the response to field stimulation, though TTX-sensitive, was partly resistant to ω -CTX (Maggi *et al.*, 1988). It seems therefore that the sensitivity to ω -CTX can vary according to the organ and species studied (Maggi *et al.*, 1988). Also, in the rat small mesenteric arteries the sensitivity of neurogenic contractions to ω -CTX appeared to be frequency-dependent, as previously described by Maggi *et al.* (1988) in the rat urinary bladder.

It has been proposed from electrophysiological studies that ω -CTX inhibits L- and N-type VOCCs in neurones from vertebrates (McCleskey *et al.*, 1987). The present study does not allow us to differentiate between channels but if we assume that ω -CTX blocks both the presynaptic L- and N-type VOCCs, the results indicate that L-type neuronal VOCCs are different from L-type muscular channels. It is well known that L-type calcium channels of vascular smooth muscle are inhibited by dihydropyridines (Godfraind *et al.*, 1986). In rat small mesenteric arteries, felodipine (0.1 μ M) inhibited the contractile response to noradrenaline by $96.3 \pm 1.3\%$ ($n = 4$) (J.A. Angus, unpublished data). In contrast, it is clear from our results and others that vascular smooth muscle VOCCs are insensitive to ω -CTX (McCleskey *et al.*, 1987; Lundy & Frew, 1988). In support of this view is the finding that no binding interactions occur between ω -CTX and dihydropyridine-related calcium antagonists (Barhanin *et al.*, 1988; Keith *et al.*, 1989). In rat sympathetic neurones, it has been established that N-type VOCCs are dominant in the evoked release of noradrenaline (Hirning *et al.*, 1988). We suggest that in sympathetic nerve terminals of rat small arteries ω -CTX-sensitive VOCCs, probably N-type VOCCs, are present and promote the release of noradrenaline, while VOCCs located in smooth muscle cells are totally ω -CTX-insensitive. A possible inhibitory action of ω -CTX on impulse propagation in nerves has recently been ruled out in a study in the vas deferens (Brock *et al.*, 1989).

Interestingly, in the present study both the e.j.ps and corresponding contractile responses were eliminated by ω -CTX (Figure 6). In a similar preparation it was previously observed that α, β -methylene ATP (10 μ M) abolished e.j.ps without affecting the accompanying contraction, whereas prazosin (0.01 μ M) blocked the force response and left e.j.ps almost unaffected (Angus *et al.*, 1988). These studies suggest that e.j.ps and the concomitant contractions could be mediated by the release of ATP and noradrenaline, respectively. The finding that ω -CTX blocked both the electrical and the mechanical processes suggests that the release of noradrenaline and possibly ATP are triggered by a similar ω -CTX-sensitive influx of calcium ions.

In summary, this study provides evidence of ω -CTX-sensitive VOCCs on sympathetic nerve terminals to the rat vasculature and demonstrates that these channels control the release of noradrenaline. We propose that ω -CTX could be a useful tool to achieve pharmacological sympathectomy when the role of the autonomic nervous system in the control of vascular tone is being studied.

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