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Effects of N-, P- and Q-type neuronal calcium channel antagonists on mammalian peripheral neurotransmission

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1 The effects of N-, P- and Q-type neuronal voltage-operated calcium (Ca^{2+}) channel antagonists on neurotransmission were determined in a range of cardiovascular and urogenital tissues, as well as the diaphragm, isolated from rat or mouse.

2 The pharmacological tools chosen were ω-conotoxin GVIA (CTX GVIA), a selective N-type Ca^{2+} channel antagonist, the P-type channel blocker (≤100 nM) ω-agatoxin IVA (AGA IVA) and ω-conotoxin MVIIC (CTX MVIIC), a non-selective antagonist of N-, P- and Q-type channels. The effects of these antagonists on nerve-mediated responses were assessed in right atria, vasa deferentia, phrenic nerve-hemidiaphragms and small mesenteric arteries.

3 Rat mesenteric artery contractile responses to perivascular nerve stimulation were concentration-dependently inhibited by CTX GVIA (1-10 nM); inhibition was 92% with 10 nM. CTX MVIIC was >100 fold less potent and only caused an inhibition of 46% at the highest concentration (1000 nM). AGA IVA (100 nM) had no effect.

4 In rat vas deferens stimulated at 0.05 Hz, CTX GVIA (10 nM) completely inhibited the twitch response and CTX MVIIC, about 100 fold less potent, caused total inhibition at 1000 nM. AGA IVA did not affect the twitch. In rat preparations stimulated at 20 Hz, a CTX GVIA-resistant (≤1000 nM) twitch response of 25% was apparent which could be blocked by 1000 nM AGA IVA or CTX MVIIC. In mouse vas deferens (20 Hz stimulation), CTX GVIA 10 nM caused an 87% inhibition of the twitch, the remainder being resistant to CTX GVIA, 100 nM. CTX MVIIC was only 10 fold less potent than CTX GVIA and completely inhibited the response at 1000 nM. AGA IVA (100 nM) inhibited the twitch by 55%.

5 The twitch response of the mouse phrenic nerve-hemidiaphragm was concentration-dependently inhibited by AGA IVA (1-100 nM); inhibition was 92% at 100 nM. CTX MVIIC was about 10 fold less potent than AGA IVA with an inhibition of 80% at 1000 nM. CTX GVIA was without effect. In the rat diaphragm preparation, AGA IVA (≤100 nM) and CTX GVIA (≤1000 nM) had little effect on the twitch response. CTX MVIIC (1000 nM) inhibited the twitch by 57%.

6 In rat and mouse right atria, sympathetic responses were concentration-dependently inhibited by CTX GVIA with almost complete block at 10-100 nM. CTX MVIIC was 100 fold less potent and caused complete inhibition at 1000 nM in the mouse preparation. AGA IVA did not affect atrial sympathetic responses.

7 These data suggest that N-type Ca^{2+} channels predominate in the control of sympathetic transmission in the mesenteric artery, vas deferens and right atrium. In the mouse vas deferens (and rat tissue at high stimulus frequency), P- and Q-type channels also mediate Ca^{2+} influx. P- and Q-type Ca^{2+} channels control neurosecretion at the motor endplate, with no role for N type channels.

Keywords: Calcium channels, N-type; calcium channels, P-type; calcium channels, Q-type; Voltage-operated calcium channels; ω-conotoxin GVIA; ω-conotoxin MVIIC; ω-agatoxin IVA; sympathetic neurotransmission; neuromuscular junction

Introduction

In the central nervous system, the calcium (Ca^{2+}) influx essential for neurotransmitter release is governed by a number of neuronal high voltage-operated Ca^{2+} channels. These channels have been subdivided into L-, N-, P- and Q-type based on differences in distribution, molecular biology, pharmacological and electrophysiological properties (Nowycky *et al.*, 1985; Miller, 1987; Hirning *et al.*, 1988; Tsien *et al.*, 1988; Llinás *et al.*, 1992; Zhang *et al.*, 1993; Olivera *et al.*, 1994). In the periphery, the role of these channels in regional neurotransmission is not well understood.

Various peptide antagonists have been shown to have subtype selectivity for these pre-junctional neuronal Ca^{2+} channels. ω-Conotoxin GVIA (CTX GVIA), a peptide isolated from the venom of the fish-hunting cone snail *Conus geographus* (Olivera *et al.*, 1984), blocks Ca^{2+} entry through N-type Ca^{2+} channels (McCleskey *et al.*, 1987). CTX GVIA can thus inhibit neurotransmitter release from nerve terminals by a pre-junctional action (Hirning *et al.*, 1988; Brock *et al.*, 1989; Pruneau & Angus, 1990a). CTX GVIA is highly selective for N-type channels and does not affect other neuronal Ca^{2+} channels, nor post-junctional L-type Ca^{2+} channels, even at concentrations four orders of magnitude higher than that required to block noradrenaline and ATP release from sympathetic nerves (Whorlow *et al.*, 1996). In many mammalian central neurones such as cerebellar granule and Purkinje cells responses to nerve depolarization are highly resistant to N- and L-type channel blockade with CTX GVIA or dihydropyridines (Llinás *et al.*, 1989; Mori *et al.*, 1991) and the P-type Ca^{2+} channel is the dominant route of Ca^{2+} entry (Llinás *et al.*, 1992; Uchitel *et al.*, 1992). ω-Agatoxin IVA (AGA IVA), a peptide isolated from the venom of the funnel web spider (Mintz *et al.*, 1992), is a relatively selective antagonist (at low concentrations <100 nM) of P-type Ca^{2+} channels. It blocks $^{45}Ca^{2+}$ influx into rat brain synaptosomes with an IC_{50} of about 20 nM (Mintz *et al.*, 1992). In the hippocampus, synaptic transmission is regulated by both N- and Q-type Ca^{2+} chan-

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nels (Wheeler *et al.*, 1994). High concentrations ($\geqslant$ 1000 nM) of AGA IVA or the non-selective peptide toxin ω-conotoxin MVIIC (CTX MVIIC) inhibit Ca^{2+} influx through Q-type channels (Zhang *et al.*, 1993; Wheeler *et al.*, 1994). CTX MVIIC inhibits N-, P- and Q-type Ca^{2+} channels with differences in relative potency and kinetics (Hillyard *et al.*, 1992; Olivera *et al.*, 1994; Wheeler *et al.*, 1994). It binds to N-type channels with an affinity 10–100 fold lower than CTX GVIA and blocks P-type channels at concentrations 100–1000 fold higher than AGA IVA with very slow on/off kinetics (Hillyard *et al.*, 1992; Monje *et al.*, 1993); CTX MVIIC and AGA IVA are equipotent at inhibiting Q-type channels (Wheeler *et al.*, 1994).

In the peripheral sympathetic nervous system, little is known about the role of P- and Q-type Ca^{2+} channels. With the availability of CTX GVIA, CTX MVIIC and AGA IVA as pharmacological tools, the relative importance of N-, P- and Q-type channels in various regions may be explored. The caveat is that CTX GVIA is the only specific tool with its clear selectivity for the N-type Ca^{2+} channel. However, there is a low nanomolar concentration range over which AGA IVA is selective for P-type channels; at higher concentrations it loses specificity. In contrast, CTX MVIIC is a non-selective neuronal Ca^{2+} channel antagonist. With these qualifications in mind, we have characterized the role of N-, P- and Q-type Ca^{2+} channels in functional neurotransmitter release in a range of mammalian isolated vascular, urogenital, neuromuscular and cardiac sympathetic preparations.

Methods

Sprague-Dawley rats (250–300 g) and Swiss white mice (30–35 g) were killed by gassing with 80% CO_2 in O_2 and exsanguination. All tissues, with the exception of rat and mouse vasa deferentia, were bathed during dissection, mounting and the experiment in a physiological salt solution (PSS) of the following composition (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$, 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylene-diaminetetracetic acid (EDTA) 0.026, glucose 5.5 and saturated with 95% O_2 and 5% CO_2. Vasa deferentia were bathed in Mg^{2+}-free PSS.

Rat isolated mesenteric arteries

Small vessels (300–400 μm diameter) were dissected from the mesenteric bed and mounted as ring preparations in a dual chamber isometric myograph (J.P. Trading, Aarhus, Denmark). Two vessels were set up in a 15 ml bath (at 37°C) and stretched to an internal circumference equivalent to 90% of the internal diameter of the vessel if it had been relaxed and under a transmural pressure of 100 mmHg (L_{100}, Mulvany & Halpern, 1977). Platinum electrodes (5 μm thick) were contained in the mounting supports of the myograph to deliver square wave field stimulation using Grass SD9/S88 stimulators (Quincy, MA, U.S.A.). Output from the transducer amplifier was recorded on a flat bed recorder (Model 320, W & W Scientific Instruments, Basle, Switzerland).

Vessels were allowed to equilibrate for 30 min following the normalisation procedure and were then maximally activated with high potassium PSS (standard PSS with an equimolar exchange of KCl for NaCl, i.e. K^+ 124 mM termed KPSS) followed by 10 μM noradrenaline (NA). Pre-junctional α_2-adrenoceptors were irreversibly blocked by a receptor protection procedure (Angus *et al.*, 1988). Vessels were first exposed to prazosin (0.1 μM) for 5 min and then benextramine (3 μM) for a further 5 min. Arteries were washed thoroughly over a period of 30 min. Electrical field stimulation was applied at 30 V (dial setting), 0.25 ms duration, 24 Hz frequency for a 3 s train every min. This stimulus train was chosen on the basis of data from Angus *et al.* (1988) where such stimulation caused contractions of 40–60% of that to KPSS. Tetrodotoxin (TTX, 0.1 μM, 1 min incubation) was added to confirm the neural mediation of responses to electrical stimulation; voltage was adjusted until contractile responses were totally abolished in the presence of TTX. Vessels were washed thoroughly to remove TTX from the bath. Three trains of control field stimulation were applied followed by 10 μM NA. Vessels were then equilibrated for 60 min (the optimal equilibration time as determined by Pruneau & Angus, 1990a) with either the lowest concentration of a peptide toxin or vehicle (0.9% saline) and responses obtained to a repeat of 3 trains of electrical stimulation as outlined above. Toxins studied were ω-conotoxin GVIA (CTX GVIA, 1–10 nM), ω-conotoxin MVIIC (CTX MVIIC, 100–1000 nM) and ω-agatoxin IVA (AGA IVA, 100 nM). Higher concentrations of the peptide toxins were added cumulatively and the preparations allowed 30 min equilibration before the responses to electrical field stimulation were reassessed.

Rat and mouse isolated vasa deferentia

Rat or mouse vasa deferentia were dissected with no attempt to remove the capsular connective tissue and set up in small (5 ml) organ baths at 37°C in Mg^{2+}-free PSS. The upper end (epididymal) was attached to an isotonic force transducer (Grass FT03C, Quincy, MA, U.S.A.) and the lower end (prostatic) threaded through two platinum electrodes (2 mm apart, 2 mm long) and tied to a fixed support. Rat and mouse tissues were initially stretched by 2 or 0.5 g force, respectively, and allowed to equilibrate for 60 min. The rat tissues were stimulated (Grass S88 stimulator) to contract (twitch) with a single electrical stimulus or a train of 20 Hz for 1 s (100 V, 0.2 ms duration) delivered every 20 s for the rat and at 20 Hz for 1 s every 20 s for the mouse preparations. Output from the transducer amplifier was recorded on a flat bed recorder (Linearcorder WR3300, Graphtec, Tokyo, Japan). The effects of vehicle (0.9% saline), CTX GVIA (1–1000 nM), CTX MVIIC (10–1000 nM) or AGA IVA (1–100 nM) were measured as the percentage reduction of the pre-drug twitch force. Each drug concentration was added cumulatively and equilibrated for 20 min before the responses to field stimulation were assessed.

Rat and mouse isolated phrenic nerve-hemidiaphragms

A portion of the diaphragm with the phrenic nerve attached was carefully dissected from rats or mice and set up in small (5 ml) organ baths at 32°C in PSS. The proximal portion of muscle was attached to an isotonic force transducer (Grass FT03C), the distal portion tied to a fixed support and the phrenic nerve threaded through a platinum electrode. Rat and mouse tissues were initially stretched by 2 or 1 g force, respectively, and allowed to equilibrate for 60 min. The diaphragm was stimulated to contract (twitch) with single electrical stimuli (4 V, 0.1 ms duration, 0.2 Hz; Grass SD9 stimulator). Output from the transducer amplifier was recorded on a flat bed recorder (Neotrace 600ZF, Neomedix, Sydney, NSW, Australia). The effects of vehicle (0.9% saline), CTX GVIA (1–1000 nM), CTX MVIIC (10–1000 nM) or AGA IVA (1–100 nM) were measured as the percentage-reduction of the pre-drug twitch force. Each drug concentration was added cumulatively and equilibrated for 30 min before the responses to nerve stimulation were assessed.

Rat and mouse isolated right atria

The right atrium isolated from rat or mouse was placed vertically on stainless steel S-shaped hooks attached to a Grass FT03C force transducer in a PSS-filled 10 ml glass-jacketed organ bath heated to 37°C. The partially stretched atrium rested against two punctate platinum electrodes protruding from the tissue holder 3 mm apart that recorded the spontaneous surface electrogram (monitored on a dual beam 10 MHz storage oscilloscope, Model T912, Tektronix, Guernsey, U.K.). This signal was amplified (Baker Medical Research

Institute (BMRI) amplifier Model 108, Prahran, Vic., Australia) and used to trigger a rate meter (BMRI Model 173). Atrial rate and force of contraction were continuously recorded on a chart recorder (Neotrace 600ZF). When required the surface electrogram signal was passed to a field pulse controller (BMRI Model 136) that triggered the start of a train of field pulses delivered via a Grass S88C dual stimulator and a pair of platinum wire field electrodes that were arranged along side and parallel to the atrium. This equipment could deliver 1–4 field pulses (2 ms duration, 100 mA) across the tissue in the atrial refractory period to avoid rhythm disturbances but allow depolarization of the autonomic varicosities and release of acetylcholine and noradrenaline (Angus & Harvey, 1981). The frequency of pulses within a single train (2–4 pulses) was set at 90 Hz. This method caused graded bradycardia within the atrial period in which the train was delivered followed by a tachycardia reaching a peak 5–10 intervals later.

To examine the sympathetic response in the absence of bradycardia, the atria were equilibrated for 1 h in the presence of atropine (1 μM). Two sets of control field pulse stimuli (1, 2 or 4 pulses in one train) were applied before adding vehicle (0.9% saline), CTX GVIA (1–100 nM), CTX MVIIC (10–1000 nM) or AGA IVA (1–100 nM). Each drug concentration was added cumulatively and equilibrated for 30 min before the responses to field stimulation were reassessed.

Drugs

Drugs used and suppliers were: ω-agatoxin IVA (AGA IVA, Peptide Institute, Osaka, Japan), atropine sulphate (Sigma, St. Louis, MO, U.S.A.), benextramine tetrachloride (Sigma), ω-conotoxin GVIA (CTX GVIA, synthesized by J.P. Flinn & R. Murphy, Department of Pharmacology, University of Melbourne, Australia, Flinn *et al.*, 1995), ω-conotoxin MVIIC (CTX MVIIC, Peptide Institute), (-)-noradrenaline bitartrate (Arterenol, Sigma), prazosin hydrochloride (Sigma) and tetrodotoxin (TTX; Calbiochem, La Jolla, CA, U.S.A.). Solutions of NA were made up fresh daily in distilled water. Prazosin and benextramine were stored as stock solutions at 4°C. TTX was stored as a stock solution at −20°C and diluted as required. The peptide toxins CTX GVIA, CTX MVIIC and AGA IVA were diluted to 0.1–1 mM in distilled water, each aliquot lyophilized and stored at −20°C. A fresh peptide toxin aliquot was made up in distilled water for each experiment.

Statistical analysis

Data are presented as mean ± 1 standard error of the mean (s.e.mean). The average s.e.mean within tissues was calculated from repeated measures analysis of variance (ANOVA) using the pooled estimate of error from the residual mean square as (error mean square/number of tissues)$^{0.5}$ after sums of squares between tissues and between peptide concentrations (or, in atria, number of electrical field pulses) had been subtracted from the total sums of squares for each treatment group (Snedecor & Cochran, 1989). These average s.e.means are described in the legends of Figures 1–4 and located on the lines shown in Figures 5 and 6 (Wright *et al.*, 1987). Responses elicited by electrical field stimulation of mesenteric arteries are expressed as a percentage of those obtained to the second set of control field stimulation (C2). Responses of vasa deferentia and hemidiaphragms are expressed as a percentage of the baseline twitch contraction. Sympathetic responses to electrical field stimulation of right atria are presented as absolute atrial rate.

For each preparation, within and between drug treatment groups, responses to electrical field stimulation were assessed by repeated measures ANOVA with Greenhouse-Geisser correction for correlation (Ludbrook, 1994), calculated by means of the statistical programme SuperANOVA 1.11 for Macintosh. Where appropriate, comparisons between two particular drug treatments were made with a *post-hoc* Tukey-Kramer test.

Values of $P<0.05$ were accepted as statistically significant.

Results

Rat isolated mesenteric arteries

The average internal diameter of the mesenteric arteries at L_{100} was 371.9 ± 9.8 μm ($n=24$). The contractions in response to electrical field stimulation were abolished in the presence of TTX (0.1 μM) indicating mediation by intramural nerves. Responses to the first set of control field stimulation (C1) were similar in all drug treatment groups, except for the CTX GVIA group where the average was significantly less than that in the vehicle group ($P<0.01$, Tukey-Kramer post-hoc test; Figure 1). Contractile responses were unaffected by vehicle administration and consistent throughout the experimental period. CTX GVIA (1–10 nM) concentration-dependently inhibited the contraction to nerve stimulation; with 10 nM, the degree of inhibition was 92 ± 2% ($n=6$; Figure 1; $P=0.0001$, repeated measures ANOVA). CTX MVIIC was more than 100 fold less potent than CTX GVIA at inhibiting nerve-mediated contractions of the mesenteric artery and at the highest concentration tested (1000 nM), this inhibition was only 45 ± 6% ($n=6$; Figure 1; $P=0.001$, repeated measures ANOVA). AGA IVA (100 nM) had no significant effect on the contraction to nerve stimulation ($n=6$; Figure 1; $P=0.25$).

Rat and mouse isolated vasa deferentia

In rat preparations stimulated with single pulses every 20 s, there was a gradual decrease in the twitch response to nerve stimulation in the vehicle treatment group over time ($P=0.003$, repeated measures ANOVA). Responses in the presence of AGA IVA (1–100 nM) were not significantly different from those in the vehicle group ($P>0.05$; Tukey-Kramer post-hoc test; Figure 2a). CTX GVIA completely inhibited the twitch response at 10 nM and CTX MVIIC was approximately 100 fold less potent, inhibiting the response completely at 1000 nM (Figure 2a). In the mouse preparation stimulated with trains of stimuli, there was also a gradual decrease in the twitch in the presence of vehicle ($P=0.0008$, repeated measures ANOVA). In contrast to its

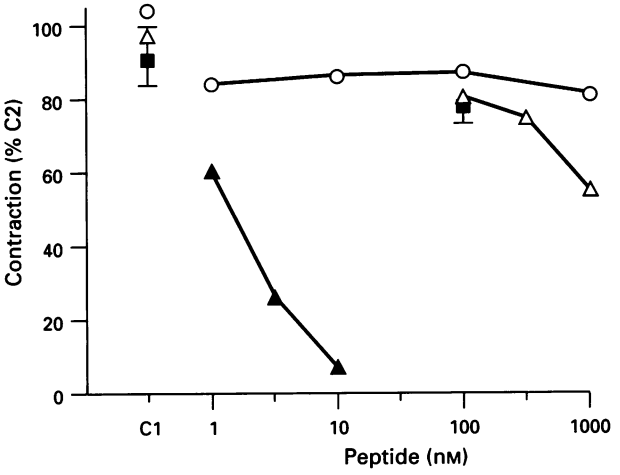

Figure 1 Effects of vehicle (0.9% saline, ○, $n=6$), ω-conotoxin GVIA (1–10 nM, ▲, $n=6$), ω-conotoxin MVIIC (100–1000 nM, △, $n=6$) or ω-agatoxin IVA (100 nM, ■, $n=6$) on contractions elicited by electrical field stimulation of rat isolated mesenteric arteries. Responses are expressed as a percentage of the contraction caused by the second control field stimulation (C2). C1 represents the effect of the first control field stimulation. Error bars on C1 (and on ω-agatoxin IVA ■) are ±1 s.e.mean (those not shown are within the symbol). For each line, the average s.e.mean from ANOVA (see Methods) was as follows: saline, ±0.9%; ω-conotoxin GVIA, ±4.9%; and ω-conotoxin MVIIC, ±2.0%.

lack of effect in rat tissues stimulated with single pulses, AGA IVA inhibited the mouse vas deferens twitch response by 55±4% at 100 nM ($n=5$; $P=0.0008$, repeated measures ANOVA; Figure 2b). CTX GVIA 10 nM caused an inhibition of the mouse vas deferens twitch of 87±4% and the residual response was resistant to higher concentrations of CTX GVIA. CTX MVIIC, more effective at inhibiting the contraction in the mouse compared to the rat preparation, was approximately 10 fold less potent than CTX GVIA. Further, CTX MVIIC (1000 nM) was able to completely inhibit the twitch response (Figure 2b).

When rat isolated vasa deferentia were stimulated at the same frequency as the mouse preparation (i.e. 20 Hz), a CTX GVIA-resistant (≤1000 nM) contraction response of approximately 25% was observed (Figure 3). With CTX GVIA (1000 nM) still present in the organ bath, this residual response was unaffected by the addition of either AGA IVA (100 nM) or CTX MVIIC (100 nM), but could be completely inhibited by 1000 nM of either peptide (Figure 3a and b, respectively).

Rat and mouse isolated phrenic nerve-hemidiaphragms

In the rat neuromuscular preparation, there was no significant change in the twitch response to nerve stimulation in the vehicle treatment group over time ($P=0.09$, repeated measures ANOVA; Figure 4a). AGA IVA (100 nM) and CTX GVIA (1000 nM) inhibited the diaphragm twitch by only 16±3 and 14±1% respectively ($P=0.009$ and 0.017 respectively, repeated measures ANOVA), but the concentration-inhibition lines were not significantly different from that in the vehicle group (Figure 4a). CTX MVIIC (1000 nM) caused an inhibition of 56±8% ($P=0.009$, repeated measures ANOVA; Figure 4a).

In the mouse isolated diaphragm, there was a small decrease of 15±1% in the twitch response to nerve stimulation in the vehicle treatment group over time ($n=4$; $P=0.0003$, repeated measures ANOVA). In the presence of CTX GVIA there was a similar decrease of 14±3% ($n=4$; $P=0.018$, repeated measures ANOVA) which was not different from vehicle treatment ($P>0.05$, Tukey-Kramer *post-hoc* test; Figure 4b). AGA IVA

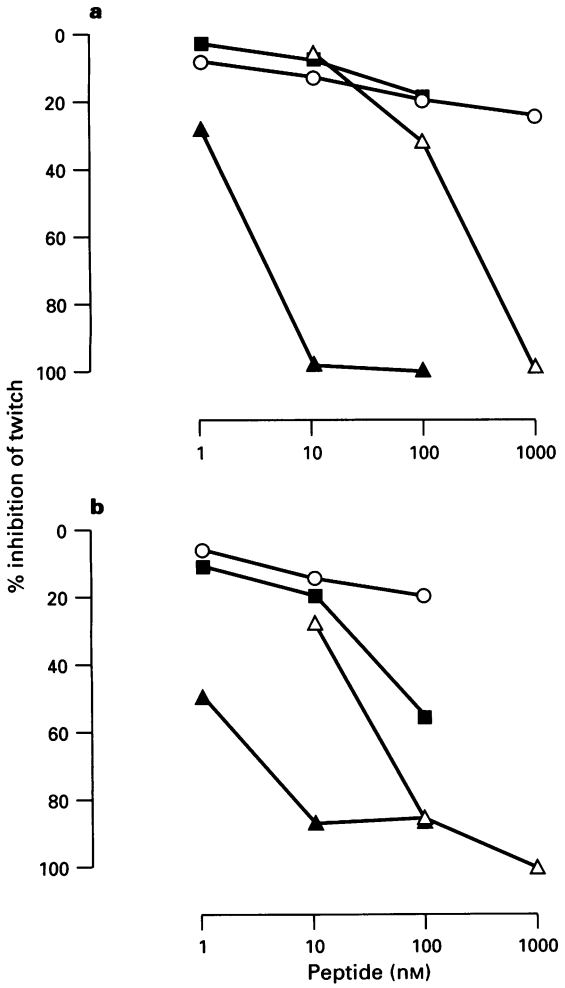

Figure 2 Effects of vehicle (0.9% saline, ○, $n=4-6$), ω-conotoxin GVIA (1–100 nM, ▲, $n=5$), ω-conotoxin MVIIC (10–1000 nM, △, $n=5$) or ω-agatoxin IVA (1–100 nM, ■, $n=5$) on the twitch response to a single pulse of electrical field stimulation of rat (every 20 s; a) and mouse (stimulated at 20 Hz for 1 s every 20 s; b) vasa deferentia. Responses are expressed as percentage inhibition of the twitch. For each line, the average s.e.mean from ANOVA (see Methods) was as follows: (a) saline, ±1.2%; ω-conotoxin GVIA, ±2.2%; ω-conotoxin MVIIC, ±4.1%; and ω-agatoxin IVA, ±1.6%; (b) saline, ±1.0%; ω-conotoxin GVIA, ±2.6%; ω-conotoxin MVIIC, ±4.0%; and ω-agatoxin IVA, ±3.3%.

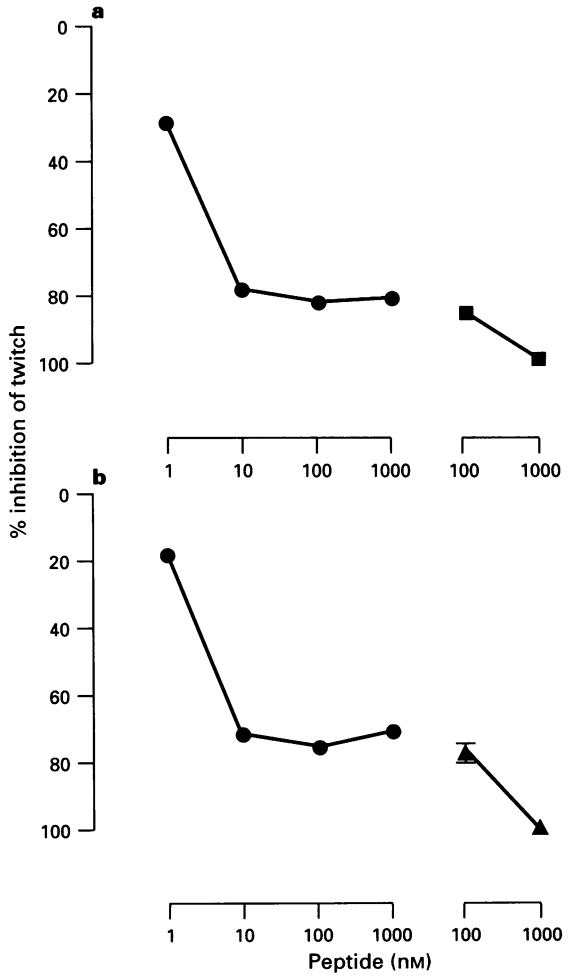

Figure 3 Effects of ω-conotoxin GVIA (●, 1–1000 nM) on the rat vas deferens contraction elicited by stronger electrical field stimulation at 20 Hz ($n=4-5$). In the presence of ω-conotoxin GVIA (1000 nM), the effects of ω-agatoxin IVA (■, 100–1000 nM; a; $n=5$) or ω-conotoxin MVIIC (▲, 100–1000 nM; b; $n=4$) on the persisting contractile responses were assessed. Responses are expressed as percentage inhibition of the control contraction. Error bars on ■ and ▲ are ±1 s.e.mean (those not shown are contained within the symbol). For each line (●), the average s.e.mean from ANOVA (see Methods) was as follows: (a) ±3.1% and (b) ±3.5%.

(1–100 nM) concentration-dependently inhibited the twitch with an almost complete inhibition of 92±3% at 100 nM ($n=5$, $P=0.0001$, repeated measures ANOVA). CTX MVIIC (1000 nM) blocked the diaphragm twitch response by 80±3% ($n=5$; $P=0.0001$, repeated measures ANOVA; Figure 4b).

Rat and mouse isolated right atria

In rat and mouse isolated right atria in the presence of atropine (1 μM), vehicle and AGA IVA (≤100 nM) were without effect on the increase in atrial rate elicited by sympathetic nerve stimulation ($P>0.05$, repeated measures ANOVA; Figures 5a and 6a). CTX GVIA 1 nM caused significant blockade of the sympathetic response in rat and mouse isolated atria ($P=0.003$ and 0.0009 respectively, repeated measures ANOVA) and by 10–100 nM the increase in rate was almost abolished (Figures 5b and 6b). The lowest concentration of CTX MVIIC (10 nM) caused significant blockade of the atrial tachycardia in mouse preparations ($P=0.021$), but did not affect responses in rat atria ($P=0.32$, repeated measures ANOVA). Higher CTX MVIIC concentrations (100–1000 nM) inhibited the tachycardia in preparations from both species but were more effective in mouse atria where 1000 nM all but abolished the response to nerve stimulation (Figures 5b(ii) and 6b(ii)). Thus, CTX MVIIC was approximately 100 fold less potent than CTX GVIA.

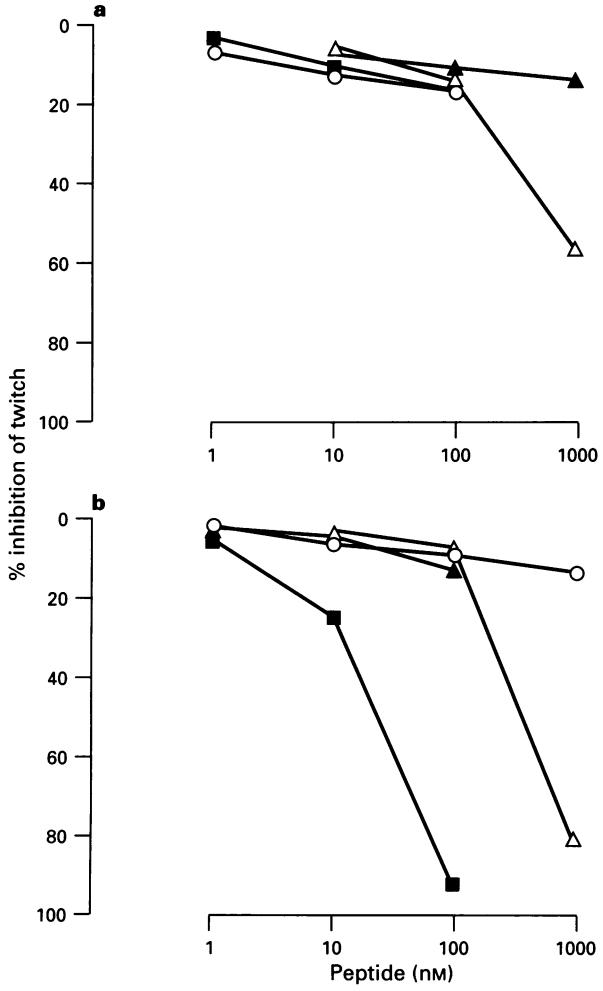

Figure 4 Effects of vehicle (0.9% saline, ○, $n=4$), ω-conotoxin GVIA (1–1000 nM, ▲, $n=4$), ω-conotoxin MVIIC (10–1000 nM, △, $n=4-5$) or ω-agatoxin IVA (1–100 nM, ■, $n=5-6$) on the twitch response elicited by electrical field stimulation of rat (a) and mouse (b) phrenic nerve-hemidiaphragm preparations. Responses are expressed as percentage inhibition of the twitch. For each line, the average s.e.mean from ANOVA (see Methods) was as follows: (a) saline, ±2.1%; ω-conotoxin GVIA, ±0.7%; ω-conotoxin MVIIC, ±4.5%; and ω-agatoxin IVA, ±1.7%; (b) saline, ±0.9%; ω-conotoxin GVIA, ±1.5%; ω-conotoxin MVIIC, ±2.7% and ω-agatoxin IVA, ±4.6%.

Discussion

The main findings of this study suggest that N-type Ca^{2+} channels are the predominant channels controlling sympathetic transmission in atria, vas deferens and small blood vessels. In vas deferens, higher frequency stimulation of rat and mouse preparations uncovers an additional role for putative Q-type channels in sympathetic neurotransmission. No role was observed for P-type channels in these three tissues. These conclusions are necessarily tentative as regards P- and Q-type channel involvement because as yet there are no peptide antagonists sufficiently selective to allow us to be more definitive. Thus the pattern of concentration-response inhibition curves in the parameters of range and location remain the best guide to interpret channel activity.

N-type Ca^{2+} channels are dominant in controlling neurotransmitter release in most sympathetically-innervated preparations that have been examined. CTX GVIA has been shown to be a selective inhibitor of sympathetic neurotransmission in the rat mesenteric artery (Pruneau & Angus, 1990a) and, even at concentrations four orders of magnitude greater than required to block N type Ca^{2+} channels, does not affect post-junctional L-type channels or α_1-adrenoceptors (Whorlow *et al.*, 1996). Blockade of sympathetic neurogenic responses with CTX GVIA has been observed in isolated preparations of rabbit ear (De Luca *et al.*, 1990; Zygmunt & Högestätt, 1993) and pulmonary (Russell *et al.*, 1990) artery, human saphenous vein (Fabi *et al.*, 1993), rat tail artery (Clasbrummel *et al.*, 1989) and canine splenic artery (Ren *et al.*, 1994). In the present study, CTX GVIA almost totally abolished contractile responses to perivascular nerve stimulation in the rat mesenteric artery and vas deferens and markedly inhibited responses to a range of stimuli in rat and mouse atria. The blood vessel and vas deferens were stimulated only at a fixed level of frequency and the contraction reduced by increasing concentrations of peptide toxin. The right atrial sympathetic assay allows families of stimulus-response curves to be generated in the presence of increasing concentrations of peptide. In the rat mesenteric artery, CTX MVIIC was at least 100 fold less potent than CTX GVIA and caused only a partial inhibition of <50% at 100 nM (AGA IVA was ineffective). This antagonist profile is consistent with neurotransmitter release being controlled by N-type Ca^{2+} channels, with no role for P- or Q-type channels (Olivera *et al.*, 1994). However, the inability of the non-selective neuronal Ca^{2+} channel antagonist, CTX MVIIC, to cause more than 50% inhibition was interesting. At a concentration of 1000 nM (~100 fold less potent than CTX GVIA), this peptide abolished responses to nerve stimulation controlled by N-type Ca^{2+} channels in other isolated preparations from the same species such as the vas deferens and right atrium (this study, see below). CTX MVIIC binds to N-type channels in many central neurone preparations with an affinity 10–100 fold less than CTX GVIA (Hillyard *et al.*, 1992; Monje *et al.*, 1993; Wheeler *et al.*, 1994), so the peptide (at 1000 nM) may have been expected to inhibit neurotransmission to a greater extent than observed in the mesenteric artery. It may be that CTX MVIIC has a lower affinity for the N-type Ca^{2+} channels in the rat mesenteric artery compared with those in other tissues (see below).

Twitch responses evoked by low frequency (0.05 Hz, single pulses every 20 s) electrical field stimulation of rat vas deferens require Ca^{2+} entry via N-type channels as they were completely inhibited by CTX GVIA (10 nM) and CTX MVIIC (1000 nM), with no effect of AGA IVA. Earlier reports of the effects of CTX GVIA on rat isolated vas deferens confirm the dominance of N-type channels in neurotransmission (De Luca *et al.*, 1990; Boot, 1994; Lundy & Frew, 1994). In the study by

Boot (1994), CTX MVIIC was found to be a poor antagonist of the twitch response, able to cause a block of only about 40% at 1000 nM; furthermore, total inhibition of the vas deferens response was achieved only with CTX GVIA at a concentration of 100 nM (10 nM causing a block of just 20%). The reason for this difference in potency of CTX MVIIC, as well as of CTX GVIA, compared with our findings is unclear. Both studies used similar stimulation parameters, although Boot used a shorter incubation time for the peptide antagonists and in our study, to maximize the twitch response to electrical stimulation, we used Mg^{2+}-free physiological salt solution (PSS) for the vas deferens experiments. The latter is unlikely to

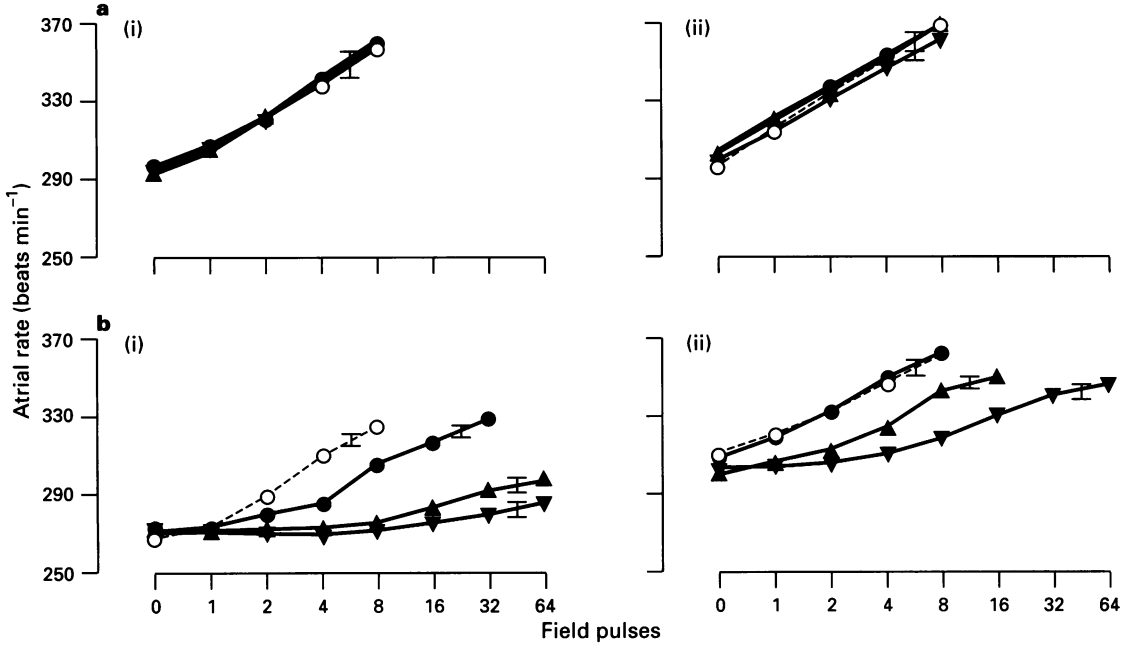

Figure 5 Effect of trains of electrical field pulses on rat isolated right atrial rate (beats min^{-1}) in the presence of 1 μM atropine: (a(i)), responses in the absence (○, dashed line) and presence of vehicle (0.9% saline, $n=5$), lines are vehicle 1 (●), 2 (▲) and 3 (▼); (a(ii)), responses in the absence (○, dashed line) and presence of 1 nM (●), 10 nM (▲) or 100 nM (▼) ω-agatoxin IVA ($n=4$). (b(i)) Responses in the absence (○, dashed line) and presence of 1 nM (●), 10 nM (▲) or 100 nM (▼) ω-conotoxin GVIA ($n=4$); (b(ii)), responses in the absence (○, dashed line) and presence of 10 nM (●), 100 nM (▲) or 1000 nM (▼) ω-conotoxin MVIIC ($n=4$). Error bars on the lines are average s.e.mean from ANOVA (see Methods).

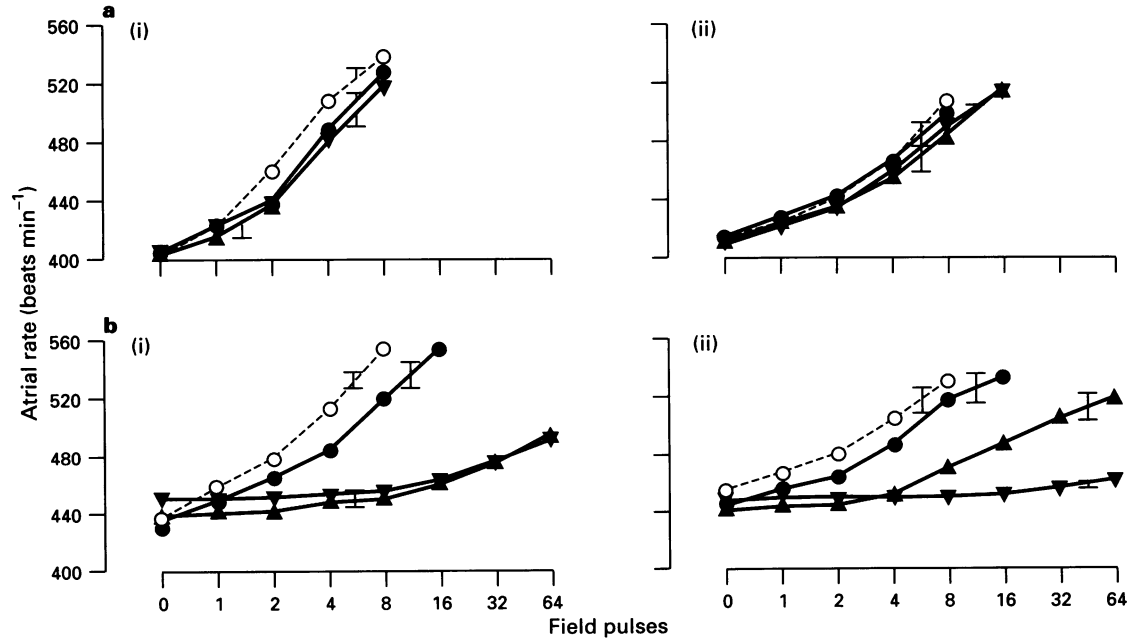

Figure 6 Effect of trains of electrical field pulses on mouse isolated right atrial rate (beats min^{-1}) in the presence of 1 μM atropine: (a(i)), responses in the absence (○, dashed line) and presence of vehicle (0.9% saline, $n=4$), lines are vehicle 1 (●), 2 (▲) and 3 (▼); (a(ii)), responses in the absence (○, dashed line) and presence of 1 nM (●), 10 nM (▲) or 100 nM (▼) ω-agatoxin IVA ($n=4$). (b(i)) Responses in the absence (○, dashed line) and presence of 1 nM (●), 10 nM (▲) or 100 nM (▼) ω-conotoxin GVIA ($n=5$); (b(ii)), responses in the absence (○, dashed line) and presence of 10 nM (●), 100 nM (▲) or 1000 nM (▼) ω-conotoxin MVIIC ($n=4$). Error bars on the lines are average s.e.mean from ANOVA (see Methods).

be the explanation for the differences in peptide toxin potency as we have examined the effects of CTX GVIA and CTX MVIIC on the vas deferens twitch response with Mg^{2+} in the PSS and found that complete inhibition was achieved with concentrations of 10 nM (pIC_{50} 8.60; $n=1$) and 1000 nM (pIC_{50} 6.76; $n=1$), respectively, data consistent with the current study. Similarly, Lundy & Frew (1994) reported complete inhibition of the isolated vas deferens twitch response with CTX GVIA 10 nM in normal PSS.

Interestingly, in mouse vas deferens stimulated at 20 Hz, 10–15% of the twitch remained in the presence of CTX GVIA 10–100 nM, whereas CTX MVIIC was only 10 fold less potent than CTX GVIA and able to cause complete inhibition at 1000 nM. Further, AGA IVA (100 nM) inhibited the twitch by as much as 55%. These data suggest that while N-type channels are the predominant route of Ca^{2+} entry in this tissue, there is also significant Ca^{2+} influx through P- and Q-type channels. When we repeated the rat vas deferens experiments using the same frequency of stimulation (20 Hz) as for the mouse tissues, a CTX GVIA-resistant component of about 25% was revealed which could be abolished by CTX MVIIC (1000 nM) or high concentrations of AGA IVA (1000 nM). Given the insensitivity of this residual response to lower concentrations of AGA IVA (i.e. 100 nM), there appears to be little role for P-type Ca^{2+} channels in the rat preparation in contrast to the mouse. Q-type channels are the most likely route of Ca^{2+} influx responsible for the CTX GVIA-resistant twitch response evident with high stimulus frequency. Zygmunt *et al.* (1993) also found that the inhibition by CTX GVIA of rabbit urethral smooth muscle responses to electrical field stimulation decreased with increasing stimulus frequencies and a CTX GVIA-resistant component of ~30% was evident at 30 Hz. More recently, Smith & Cunnane (1996) have reported a CTX GVIA-resistant component of neurotransmitter release from guinea-pig isolated vas deferens when the tissues were stimulated at >1 Hz. The Ca^{2+} channel subtype(s) controlling the remaining response to nerve stimulation in each case was not determined (Zygmunt *et al.*, 1993; Smith & Cunnane, 1996).

Sympathetic neurotransmission in rat and mouse spontaneously beating right atria is controlled by N-type Ca^{2+} channels with no evidence for the involvement of P- and Q-type channels. CTX GVIA caused an almost complete block of the increase in atrial rate following sympathetic nerve stimulation (in the presence of muscarinic receptor inhibition) and CTX MVIIC was 100 fold less potent. CTX MVIIC appeared to be slightly more effective in the mouse compared with the rat tissue causing complete inhibition at 1000 nM. The P-type antagonist, AGA IVA, had no effect. In guinea-pig isolated left atria, a similar antagonist profile has been reported with block of sympathetic transmitter release by CTX GVIA, as well as CTX MVIIC at 100 fold higher concentrations, and no effect of AGA IVA (Vega *et al.*, 1995). Hong & Chang (1995) similarly found a predominant role for N-type channels in sympathetic transmitter release in guinea-pig right and left atria; however, CTX GVIA was less potent causing complete inhibition at 1 μM, equipotent with CTX MVIIC.

At the neuromuscular junction P-, and perhaps Q-, type Ca^{2+} channels control neurosecretion, with no role for N-type channels. In the mouse, the twitch response of the phrenic nerve-hemidiaphragm preparation was almost completely abolished by low concentrations of AGA IVA consistent with a P-type channel classification for the principal ion channel in this preparation (Llinás *et al.*, 1992; Uchitel *et al.*, 1992; Protti & Uchitel, 1993; Olivera *et al.*, 1994). CTX MVIIC (1000 nM), about 10 fold less potent than AGA IVA, caused an inhibition of 80% whereas CTX GVIA was without effect. In the rat diaphragm however, P-type channels do not seem to control transmitter release as AGA IVA had little effect on the twitch response. CTX GVIA did not cause significant inhibition ruling out an involvement of N-type channels, but CTX MVIIC (1000 nM) did inhibit a major component (57%) of the twitch response. This suggests that Q-type Ca^{2+} channels may contribute to neurosecretion in the rat. CTX GVIA has been shown by others to have no effect on transmission at the neuromuscular junction in the rat and mouse (De Luca *et al.*, 1991; Protti & Uchitel, 1993). However other workers have demonstrated a significant blockade of neuromuscular transmission by CTX GVIA at low nanomolar concentrations in the rat, both *in vitro* and *in vivo* (Rossoni *et al.*, 1994). In the conscious rabbit, acute or chronic intravenous administration of CTX GVIA at sympatholytic doses has no discernible respiratory or other neuromuscular effects (Pruneau & Angus, 1990b; Wright & Angus, 1995), suggesting that this peptide does not affect the neuromuscular junction in this species.

In conclusion, N-type Ca^{2+} channels are the major route of Ca^{2+} influx essential for mammalian peripheral sympathetic neurotransmission. However, there is a population of putative P- and Q-type Ca^{2+} channels that subserve Ca^{2+} entry in the event of N-type channel blockade and when high frequency of nerve stimulation is present. Whether co-transmitters such as neuropeptide Y or ATP are differentially sensitive to N-, P- or Q-type Ca^{2+} channel blockade needs further work. At the neuromuscular junction, P-, and perhaps Q-, type Ca^{2+} channels control neurosecretion. A more definitive classification of the role of these and other putative neuronal Ca^{2+} channel subtypes in peripheral neurotransmission awaits the availability of more selective antagonists.

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