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Differential effects of voltage-dependent Ca^{2+} channels on low and high frequency mediated neurotransmission in guinea-pig ileum and rat vas deferens

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Abstract

The ω-conotoxins GVIA, MVIIA, MVIIC and SVIB reduced in a concentration-dependent manner the low frequency electrically stimulated twitch response of the guinea-pig ileum and rat vas deferens. The relative activities of the conotoxins showed some difference between the two preparations in that for ileum it was MVIIA = GVIA > MVIIC = SVIB and for the vas deferens it was MVIIA > GVIA ≫ SVIB > MVIIC. High frequency electrical stimulation of both preparations resulted in a neurally-mediated ω-conotoxin GVIA resistant component that was sensitive to high concentrations of either ω-conotoxin MVIIC (300 nM–1 μM) or ω-agatoxin IVA (300 nM–1 μM) but not to ω-conotoxin MVIIA. Lower levels of either ω-conotoxin MVIIC or ω-agatoxin IVA (30–100 nM) failed to significantly affect the ω-conotoxin GVIA resistant component. This ω-conotoxin GVIA resistant component was large in the ileal preparation comprising 30–40% of the maximal response at 20 Hz but relatively small (10%) in the vas deferens. These studies revealed that the N-type voltage-dependent calcium channel (VDCCs) exclusively controls neurotransmission during low frequency stimulation but at higher frequencies there is an additional non-adrenergic, non-cholinergic (NANC) neurotransmission that appears to be regulated via Q-type VDCC. © 1997 Elsevier Science B.V.

Keywords: Ca^{2+} channel, voltage-dependent; ω-Conotoxin GVIA; ω-Conotoxin MVIIA; ω-Conotoxin MVIIC; ω-Conotoxin SVIB; ω-Agatoxin IVA; Neurotransmission

1. Introduction

Synaptic transmission is dependent upon the entry of calcium ions (Ca^{2+}) through presynaptic voltage dependent calcium channels (VDCCs) (Mulkey and Zucker, 1991). Several types of VDCCs have been identified within the central nervous system based on their electrophysiological and pharmacological properties and have been designated L, N, P, Q, R and T (Tsien et al., 1988; Zhang et al., 1993). During these studies several toxins were used as ligands to define the pharmacology of the channel subtypes and these have been used in this study. Omega-conotoxin GVIA, MVIIA and SVIB are selective blockers of N-type VDCCs; ω-agatoxin will block P-type at low concentrations (< 30 nM) and Q-type at higher concentrations (> 100 nM). Omega-conotoxin MVIIC will block both N-type and Q-type VDCCs.

The release of neurotransmitters and neurotransmission within the peripheral cholinergic and sympathetic autonomic system is particularly sensitive to ω-conotoxin GVIA, a selective N-type VDCC inhibitor (Hirning et al., 1988; De Luca et al., 1990; Lundy and Frew, 1993, 1994). Recently it has been shown that Q-type VDCCs may also have a functional role in maintaining neurotransmission in the non-adrenergic non-cholinergic system within the rat bladder (Frew and Lundy, 1995) and within the lamina propria of the rabbit urethra (Zygmunt et al., 1995).

We had previously shown that neurotransmission in the guinea-pig ileum and rat vas deferens in response to low frequency electrical field stimulation was particularly sensitive to ω-conotoxin GVIA (Boot, 1994). These studies have now been extended to include the effects of other ω-conotoxins on low frequency stimulation and the effects of ω-conotoxin GVIA, MVIIC and ω-agatoxin IVA on higher frequency stimulation of these preparations.

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2. Materials and methods

2.1. Field stimulated guinea-pig ileum

Short segments (2 cm) of terminal ileum were suspended between parallel silver electrodes in a 10 ml organ bath containing Tyrodes solution at 37°C aerated with 95% oxygen and 5% CO_2. The composition of the Tyrode solution used in these studies was (mM) NaCl 137; KCl 2.7; $MgCl_2$ 1; $CaCl_2$ 1.8; NaH_2PO_4 0.2; $NaHCO_3$ 12; and glucose 5.5. The ileum was equilibrated for 1 h under a maintained resting tension of 1 g.

2.1.1. Low frequency field stimulation

The preparation was stimulated at 0.1 Hz with a rectangular pulse of 0.1 ms duration and supramaximal voltage for periods of 10 min and the contractions were recorded isometrically. Omega-conotoxins were added 1 min after the start of each stimulation period and at the end acetylcholine (50 nM) was added to the bath to determine whether postsynaptic acetylcholine receptors had been affected by the toxin pretreatment. After each concentration of toxin the preparation was washed for 5 min and then rested for a further 5 min.

2.1.2. High frequency field stimulation

The preparation was stimulated in trains of 0.5 ms pulses at ascending frequencies of 1, 5, 10 and 20 Hz for 5 s every 1 min at supramaximal voltage (25 V). Three consecutive contracture responses were elicited at each frequency level. At the end of each stimulation sequence the preparation was washed and rested for 15 min. The toxins were added 5 min before the start of each stimulation period. This pretreatment time was chosen as previous studies had shown that at concentrations greater than 100 nM this was sufficient (Boot, 1994). At lower concentrations, in a manner similar to previous studies (Boot, 1994) a preincubation time of 15 min was used in some experiments. However, no additional inhibitory effect was observed so the 5 min preincubation time was used in the remainder of the studies.

2.2. Field stimulated rat vas deferens

Vasa deferentia were removed from adult rats and suspended in Krebs solution under similar conditions to that described for ileum. The Krebs (Hukovic) had the following composition (mM) NaCl 113; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; and glucose 11.

2.2.1. Low frequency field stimulation

The preparation was stimulated at 0.05 Hz with a rectangular pulse of 0.1 ms duration and supramaximal voltage (25 V) for periods of 10 min and contractions recorded as above. Different concentrations of ω-conotoxins were added 1 min after the start of the electrical stimulation and at the end, noradrenaline (5×10^{-6} M) was added to assess effects on postsynaptic adrenoreceptors.

The preparation was washed and rested as previously described.

2.2.2. High frequency field stimulation

A similar paradigm to the ileum was used. In these studies, paired vas deferens were used whenever possible to evaluate pharmacological differences between the toxins.

The inhibitory effects were calculated as a % of the consistent responses obtained at the start of each experiment before the addition of ω-conotoxins. The results are expressed as arithmetic means $\pm$ S.E.M.. The IC_{50} values were calculated by linear regression analysis.

Omega-conotoxin GVIA, MVIIA and MVIIC, were purchased from Bachem U.K. Omega-agatoxin IVA and ω-conotoxin SVIB were purchased from Peptide Inst. U.K.

3. Results

3.1. Guinea-pig ileum

3.1.1. Low frequency field stimulation

Low frequency field stimulation produced consistent twitch responses over the 10 min stimulatory period. All ω-conotoxins (1–100 nM) produced a concentration-dependent inhibition (> 80%) of the twitch response. The inhibitory effects of ω-conotoxin MVIIA, ω-conotoxin MVIIC and ω-conotoxin SVIB could be largely reversed by washing whereas the effects of ω-conotoxin GVIA were unaffected. The effective rank order by inspection was MVIIA = GVIA > SVIB = MVIIC with IC_{50} values of 4 8 ± 0.31, 8.8 ± 2.3, 25.7 ± 4.1 and 34.4 ± 7.4 nM respectively (Fig. 1A).

Omega-agatoxin IVA (1 μM) had no effect upon the electrically-induced twitch responses.

None of the ω-conotoxins (100 nM) affected the exogenously added acetylcholine response.

Atropine (1 μM) completely blocked the electrically induced twitch response and the exogenously added acetylcholine response, whereas tetrodotoxin (1 μM) completely blocked the twitch response but had no effect upon the added acetylcholine response.

3.1.2. High frequency field stimulation

Three consistent responses were generated at each frequency (1, 5, 10 and 20 Hz). All ω-conotoxins (300 nM) effectively produced maximal inhibition (> 90%) of the response at 1 Hz that decreased as the frequency increased, leaving a residual 30–50% unaffected by toxin pretreatment at 20 Hz.

Tetrodotoxin (1 μM) reduced these higher (5, 10, 20

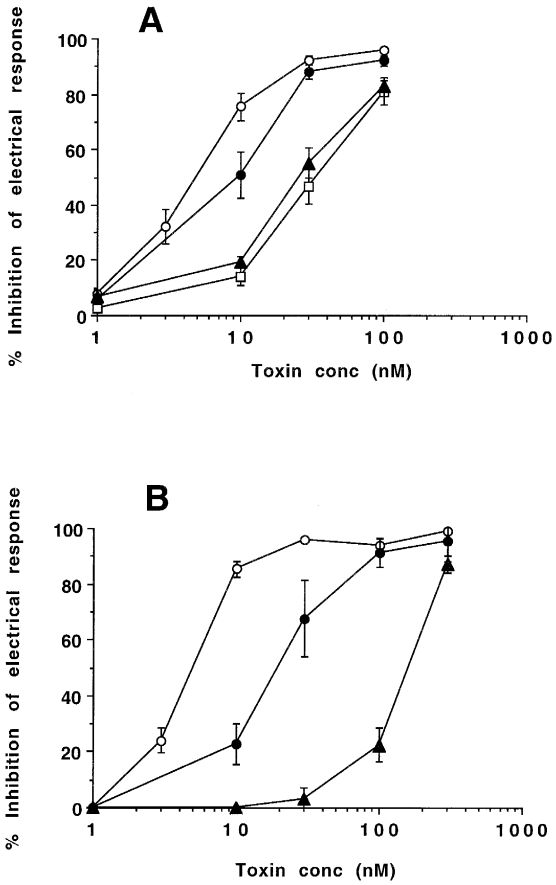

Fig. 1. The concentration-dependent inhibitory effects of ω-conotoxin GVIA –●–, ω-conotoxin MVIIA –◦–. ω-conotoxin SVIB –▲– and ω-conotoxin MVIIC –□– on electrically-induced twitch response of the guinea-pig ileum (A). The lower panel (B) shows the inhibitory effects of ω-conotoxin GVIA –●– ω-conotoxin MVIIA –◦–. ω-conotoxin SVIB –▲– on the electrically induced responses of the rat vas deferens. Omega conotoxin MVIIC only inhibited 18±5% at 300 nM. Each point represents the mean±S.E.M. ($n=4$) expressed as a percentage of the maximal effect.

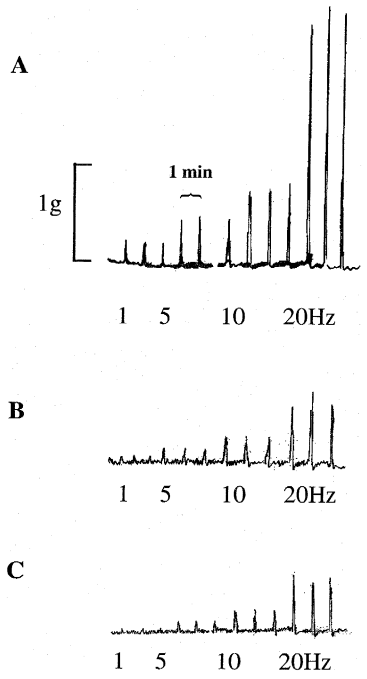

Fig. 2. The effect of ω-conotoxin GVIA on high frequency stimulated neurotransmission in the guinea pig ileum. (A) The preparation was stimulated with trains of 0.5 ms pulses at ascending frequencies of 1, 5, 10 and 20 Hz for 5 s every 1 min at supramaximal voltage (25 V). Three consecutive contracture responses were elicited at each frequency level. (B) The effect of ω-conotoxin GVIA (300 nM) added 5 min prior to further sequence of electrical stimulation. (C) The effect of further ω-conotoxin GVIA (1 μM).

Hz) frequency-induced twitch responses by 95%. Atropine (1 μM, 3 μM) produced a complete block at 1 Hz but like the ω-conotoxin as the frequency increased the inhibitory effect was reduced such that at 20 Hz a partial inhibitory effect of 60% was achieved. The addition of propranolol and prazosin (10 μM) had no additional effect on the partial atropine block at 20 Hz.

The persistent, selective N-type VDCC inhibitor ω-conotoxin GVIA (300 nM) was used in all subsequent experiments to study the pharmacology of the ω-conotoxin GVIA resistant component at the higher frequencies. This ω-conotoxin GVIA resistant component was not reduced by the further addition of either ω-conotoxin GVIA (300 nM, 1 μM) or ω-conotoxin MVIIA (300 nM) (Fig. 2A, Fig. 3). However the addition of either ω-conotoxin MVIIC or ω-agatoxin IVA (300 nM, 1 μM) did reduce the ω-conotoxin GVIA resistant response in a concentration and statistically significant manner, resulting in almost complete inhibition of the 20 Hz response at the higher concen-

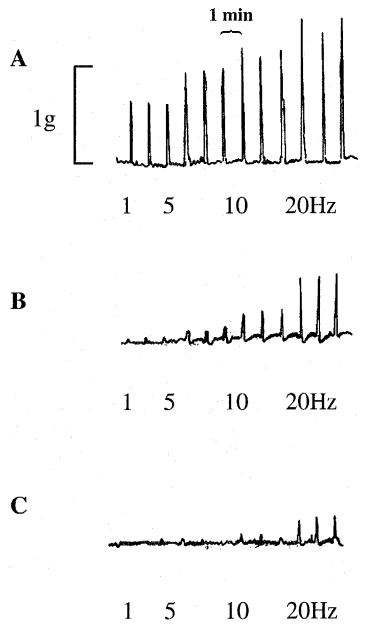

Fig. 3. The effect of ω-conotoxin GVIA and ω-conotoxin MVIIC on high frequency stimulated neurotransmission in the guinea pig ileum. (A) The control responses were obtained as described in Fig. 2. (B) The effect of added ω-conotoxin GVIA (300 nM) added 5 min prior to further sequence of electrical stimulation. (C) The effect of further ω-conotoxin MVIIC (300 nM).

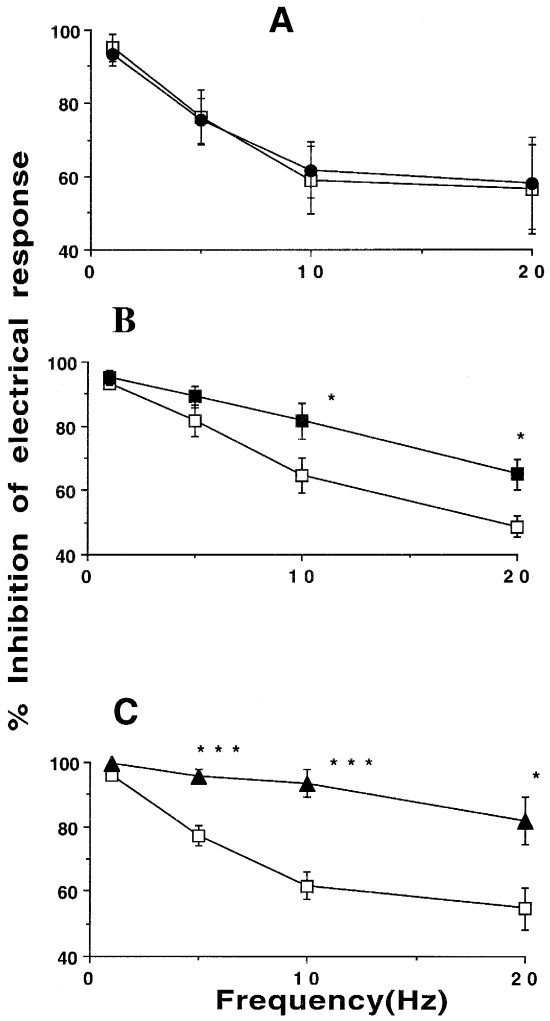

Fig. 4. The effect of ω-conotoxin GVIA and ω-conotoxin MVIIC on the ω-conotoxin GVIA (300 nM) (– □ –) resistant neurotransmission of guinea pig ileum. (A) The effect of additional ω-conotoxin GVIA 1 μM (– ● –). (B and C) The effect of additional ω-conotoxin MVIIC 300 nM (– ■ –) and 1 μM (– ▲ –). Each point represents the mean ± S.E.M. ($n = 4–6$) expressed as a percentage inhibitory effect of the control response. Statistical significance was calculated using un-paired *t*-test ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$).

tration of 1 μM (Fig. 4).

At lower concentrations, neither ω-conotoxin MVIIC (100 nM) nor ω-agatoxin IVA (100 nM) failed to significantly affect the conotoxin GVIA resistant component.

3.2. Rat vas deferens

3.2.1. Low frequency field stimulation

Omega-conotoxins MVIIA, GVIA, SVIB all exhibited a concentration-dependent inhibition of the twitch responses produced at 0.05 Hz (Fig. 1B).

The rank order of potency in this preparation corresponded to MVIIA > GVIA > SVIB ≫ MVIIC with IC50 values of 5.2 ± 1.4, 19.4 ± 5.6 and 163 ± 9.7 nM respectively. Omega-conotoxin MVIIC (300 nM) had only a modest (18%) inhibitory effect and ω-agatoxin IVA (300 nM) had no effect on these low frequency twitch responses.

None of the ω-conotoxins affected responses of the exogenously added noradrenaline.

Tetrodotoxin (1 μM) completely blocked the electrically evoked response without having any effect upon the responses to exogenous noradrenaline.

A combination of propranolol (10 μM)), prazosin (10 μM) and the purinergic antagonist suramin (100 μM) completely blocked the electrically evoked twitch response.

3.2.2. High frequency field stimulation

Omega-conotoxin GVIA (300 nM) and ω-conotoxin MVIIA (300 nM) were very effective in blocking the high

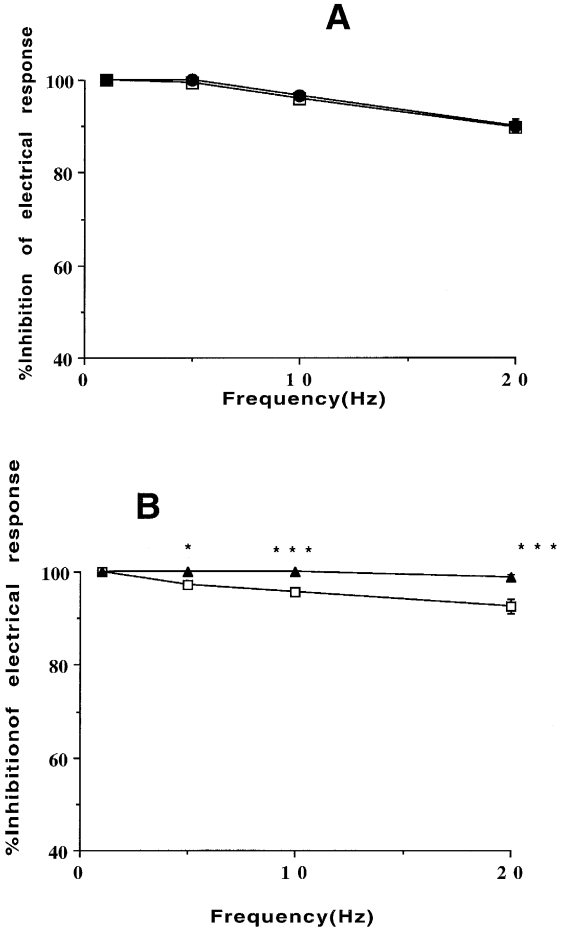

Fig. 5. The effect of ω-conotoxin GVIA and ω-conotoxin MVIIC on the ω-conotoxin GVIA (300 nM) (– □ –) resistant neurotransmission of rat vas deferens. (A) The effect of additional ω-conotoxin GVIA 1 μM (– ● –). (B) The effect of additional ω-agatoxin IVA 1 μM (– ▲ –). Each point represents the mean ± S.E.M. ($n = 4–6$) expressed as a percentage inhibitory effect of the control response. Statistical significance was calculated using un-paired *t*-test ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$).

frequency twitch responses. There was a slight fall in inhibitory effectiveness at 20 Hz with 10% of the response unaffected. There was no further reduction of this refractory component upon the addition of further ω-conotoxin GVIA (1 μM) (Fig. 5A).

Omega-conotoxin SVIB (300 nM) was less effective in inhibiting the high frequency responses, producing a 40% block at 1 Hz which was only slightly reduced across the frequency range to 34% at 20 Hz. Omega-conotoxin MVIIC and ω-agatoxin IVA appeared different from ω-conotoxin GVIA and ω-conotoxin MVIIA in that although they produced only a slight inhibitory effect at 1 Hz (25% and 12%, respectively) this was maintained across the frequency range, there was no subsequent reduction of inhibitory effect with increasing frequency. In this preparation, even at the highest frequency, the ω-conotoxin GVIA resistant component was relatively small (10–15%), nevertheless the further addition of either ω-conotoxin MVIIC (300 nM, 1 μM) (Fig. 5B) or ω-agatoxin IVA (1 μM) (unlike ω-conotoxin GVIA) resulted in a complete block at the higher frequencies. Data for ω-agatoxin IVA not shown as it was identical to ω-conotoxin MVIIC.

Tetrodotoxin (1 μM) gave complete inhibition of responses at all frequencies.

A combination of atropine (3 μM), propranolol (10 μM)), prazosin (10 μM) and suramin (100 μM) failed to block completely the electrically evoked twitch response.

4. Discussion

At low frequency field stimulation, all the ω-conotoxins (100 nM) produced a 90–100% block of the electrically induced contractions of the ileum with a rank order of potency corresponding to ω-conotoxin MVIIA = GVIA > SVIB = MVIIC. However ω-conotoxin MVIIC was less effective than previously reported (Boot, 1994). We have no explanation for this discrepancy except that we have noticed when using ω-conotoxin MVIIC as an internal standard in other biochemical tests that there were batch variations in potency and stability. This not withstanding the qualitative differences previously reported that ω-conotoxin MVIIC is less effective compared to the other ω-conotoxins on the rat vas deferens relative to the guinea-pig ileum still exists. This difference in relative potencies between the two preparations may indicate that different isoforms of the N-type VDCC exist and that ω-conotoxin MVIIC may be used to discriminate between them.

The ω-conotoxins selectively inhibited the field stimulated twitch responses without affecting the post synaptic responses to either acetylcholine on guinea-pig ileum or noradrenaline on the vas deferens preparations confirming that all the ω-conotoxins studied reduced presynaptic neurotransmitter release.

At higher frequencies on the ileum, all the ω-conotoxins were less effective in a frequency dependent manner. In these studies tetrodotoxin was effective at all frequencies confirming that the contractions were neurally mediated. The ω-conotoxin GVIA resistant component at 20 Hz that amounted to 30–50% of the response was not affected by a combination of atropine, propranolol and prazosin indicating that this GVIA resistant component appears to correspond to a non-adrenergic, non-cholinergic (NANC) neurotransmission. The further addition of either ω-conotoxin GVIA or ω-conotoxin MVIIA (selective N-type VDCC inhibitors) failed to reduce the ω-conotoxin GVIA resistant component further. However, this NANC, ω-conotoxin GVIA resistant component could be blocked in a concentration dependent-manner by the addition of either ω-conotoxin MVIIC (an N, Q, P-type VDCC inhibitor) or ω-agatoxin IVA (a P/Q type VDCC inhibitor). Omega-agatoxin IVA inhibits P-type VDCCs at low concentrations (Mintz et al., 1992), however, in this study higher concentrations comparable to those required to rapidly block Q-type VDCCs (Sather et al., 1993; Randall et al., 1993) were required to affect the ω-conotoxin GVIA resistant component. These studies indicate that in the guinea-pig ileum, low frequency-mediated neurotransmission is essentially cholinergic and that the presynaptic release of acetylcholine is controlled exclusively via N-type VDCCs. At higher frequencies in this preparation an additional NANC component is recruited which is refractory to N-type VDCC blockers but sensitive to Q-type VDCC blockers.

The ω-conotoxin GVIA resistant component at higher frequencies on the rat vas deferens was much smaller (10%) but was blocked by the further addition of relatively high concentrations of ω-conotoxin MVIIC or ω-agatoxin IVA (> 300 nM). The lack of effect by agatoxin IVA at lower concentrations, a P-type selective concentrations (Mintz et al., 1992) on either preparation agrees with the findings of Lundy and Frew (1994) that the P-type VDCC if present, plays no significant role in peripheral autonomic neurotransmission. The inhibition of the NANC-mediated neurotransmission by higher concentrations of ω-conotoxin MVIIC and ω-agatoxin IVA supports a role for Q-type VDCC in the autonomic system and complements the findings of Frew and Lundy (1995). During the final preparation of this manuscript a report by Waterman (1997) showed that low frequency electrical stimulation of mouse vas deferens was completely blocked by ω-conotoxin GVIA and that higher frequencies responses were less responsive to ω-conotoxin GVIA but were blocked by ω-conotoxin MVIIC. Her finding also suggested that N-, P- and Q-type VDCCs were involved in the release of noradrenaline and adenosine 5′-triphosphate and that there was no functional separation of neurotransmitter release with the frequency of electrical stimulation used in the experiments. In conclusion there is now sufficient data across a range of innervated preparations to indicate a role for N- and Q-type VDCCs in neurotransmitter release from autonomic neurones.

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