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Role of N-type calcium channels in autonomic neurotransmission in guinea-pig isolated left atria

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1 Calcium entry via neuronal calcium channels is essential for the process of neurotransmission. We investigated the calcium channel subtypes involved in the operation of cardiac autonomic neurotransmission by examining the effects of selective calcium channel blockers on the inotropic responses to electrical field stimulation (EFS) of driven (4 Hz) guinea-pig isolated left atria. In this tissue, a previous report (Hong & Chang, 1995) found no evidence for N-type channels involved in the vagal negative inotropic response and only weak involvement in sympathetic responses.

2 The effects of cumulative concentrations of the selective N-type calcium channel blocker, ω-conotoxin GVIA (GVIA; 0.1–10 nM) and the non-selective N-, P/Q-type calcium channel blocker, ω-conotoxin MVIIC (MVIIC; 0.01–10 nM) were examined on the positive (with atropine, 1 μM present) and negative (with propranolol, 1 μM and clonidine, 1 μM present) inotropic responses to EFS (eight trains, each train four pulses per punctate stimulus).

3 GVIA caused complete inhibition of both cardiac vagal and sympathetic inotropic responses to EFS. GVIA was equipotent at inhibiting positive (pIC_{50} 9.29 $\pm$ 0.08) and negative (pIC_{50} 9.13 $\pm$ 0.17) inotropic responses. MVIIC also mediated complete inhibition of inotropic responses to EFS and was 160 and 85 fold less potent than GVIA at inhibiting positive (pIC_{50} 7.08 $\pm$ 0.10) and negative (pIC_{50} 7.20 $\pm$ 0.14) inotropic responses, respectively. MVIIC was also equipotent at inhibiting both sympathetic and vagal responses.

4 Our data demonstrates that N-type calcium channels account for all the calcium current required for cardiac autonomic neurotransmission in the guinea-pig isolated left atrium.

Keywords: Cardiac autonomic neurotransmission; N-type calcium channel; inotropic; atria; sympathetic; vagus; ω-conotoxin GVIA; ω-conotoxin MVIIC

Abbreviations: C2, second control electrical field stimulus; DHPs, dihydropyridines; EFS, electrical field stimulation; GVIA, ω-conotoxin GVIA; HVA, high voltage-activated; MVIIC, ω-conotoxin MVIIC; NANC, non-adrenergic, non-cholinergic; NPY, neuropeptide Y; pIC_{50}, $-$log(molar concentration of peptide eliciting 50% maximum inhibition); VOCCs, voltage operated calcium channels

Introduction

Electrically-evoked neurotransmitter release is critically dependent on the influx of extracellular calcium across the neuronal cell membrane (Mulkey & Zucker, 1991). Under depolarizing conditions, calcium enters the cell through selective pores within the cell membrane. Collectively, these cationic pores are referred to as voltage-operated calcium channels (VOCCs) and may be classified as low voltage activated (T-type) or high voltage activated (HVA) calcium channels according to their unique electrophysiological properties (Nowycky *et al*., 1985). The HVA calcium channels are further subdivided into two classes, based on their sensitivity to the 1,4-dihydropyridine (DHP) group of compounds. The DHP-sensitive, HVA calcium channel is also referred to as the L-type VOCC due to its *l*ong inactivation kinetics and *l*arge unitary conductance (Nowycky *et al*., 1985). VOCCs that are DHP-insensitive and have intermediary inactivation kinetics, being *n*either long nor transient have been named N-type VOCCs (Nowycky *et al*., 1985). It now appears that the N-type calcium channel is located exclusively on neurons where their function is thought to be in mediating neurotransmitter release (Olivera *et al*., 1994) and as such, may also adopt the N- prefix for neuronal. N-type calcium channels are blocked by nanomolar concentrations of a peptide ω-conotoxin GVIA (GVIA), a fraction of venom isolated from the fish hunting cone snail, *Conus Geographus* (Olivera *et al*., 1984). GVIA is a highly selective ligand for the N-type calcium channel. At concentrations 10,000 times that required to inhibit sympathetic neurotransmission, GVIA has no effect at L-type calcium channels mediating vascular contractile responses, nor other neuronal calcium channels (Whorlow *et al*., 1996). It is on the basis of sensitivity to GVIA that the existence of this N-channel in a particular mammalian system can be inferred (Olivera *et al*., 1994; Lundy & Frew, 1996). P-type calcium channels were first described in cerebellar Purkinje neuron cell bodies and are resistant to both DHPs and ω-CTX GVIA (Llinas *et al*., 1989). These currents are blocked by low concentrations (IC_{50} $\sim$1 nM) of another ω-toxin, ω-agatoxin IVA, isolated from the venom of the American funnel web spider, *Agelenopsis aperta* (Llinas *et al*., 1989). Electrophysiologically, the P-type current resembles N-type current (Llinas *et al*., 1989; Mintz & Bean, 1993). Therefore, the major criterion differentiating these two channels is a pharmacological one, i.e. the lack of inhibition of the P-type channel by ω-CTX GVIA and selective inhibition by ω-agatoxin IVA. Thus, the L-, N- and P-type HVA calcium channels are all able to be defined with selective pharmacological tools. There is, however, evidence for the existence of other calcium channels that are not

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defined by current pharmacological reagents, but possess unique electrophysiological properties of their own. These include the Q-type (Zhang *et al*., 1993) O-type (Adams *et al*., 1993) and R-type (Zhang *et al*., 1993) calcium currents.

The 36 amino acid peptide co-transmitter, neuropeptide Y (NPY), has been shown to selectively inhibit presynaptic N-type calcium channels in co-cultures of sympathetic neurons and cardiac myocytes *via* an inhibitory G-protein (Toth *et al*., 1993). More recently, by utilising receptor-selective NPY analogues, McDonald *et al*. (1995) demonstrated that the subtype of NPY receptor directly linked to inhibition of N-type calcium channel was a Y_2 receptor. However, others have provided evidence to suggest that NPY Y_1 receptors may also couple to inhibition of N-type calcium channels (McQuiston *et al*., 1996). In the guinea-pig isolated left atrium, Hong & Chang (1995) reported that ω-conotoxin GVIA was ineffective at inhibiting the vagally-mediated, negative inotropic response to trains of electrical field stimulation (Hong & Chang, 1995). This would suggest that release of acetylcholine from vagal nerve varicosities is dependent on calcium entry *via* non-N-type calcium channels. In the same tissue, Serone & Angus (1999) found that NPY was a potent inhibitor of vagal nerve-mediated decreases in force of contraction. This prejunctional inhibition of vagal neurotransmission was mediated *via* a Y_2 receptor (Serone & Angus, 1999). Therefore, the work of Hong & Chang (1995) together with our studies of NPY would suggest that the Y_2 receptor on vagal nerve terminals is coupled to a calcium channel other than the N-type calcium channel.

The aim of this study was to examine the calcium channel subtypes involved in the regulation of cardiac vagal and sympathetic neurotransmission in the guinea-pig isolated left atrium. The results presented here show, in contrast to the previous report of Hong & Chang (1995), that N-type calcium channels do account for the majority of calcium current involved in both cardiac vagal and sympathetic autonomic neurotransmission.

Methods

General

Guinea-pigs of either sex (594 ± 20 g, range $395-860$ g, $n=30$) were killed by cervical dislocation. The heart was rapidly removed, placed in oxygenated Krebs' solution (see below) maintained at 37°C. The left atrium was then dissected free of surrounding vessels and connective tissue and suspended vertically on stainless steel S-shaped hooks attached to a Grass FT03C force transducer. Atria were maintained at a resting force of approximately 1 g. The Krebs' solution had the following composition (in mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, EDTA 0.026 and glucose 11.

Atria rested against two punctate platinum electrodes (3 mm apart) and were stimulated with square wave pulses (4 Hz, 2 ms, twice threshold voltage) delivered *via* a Grass S88C dual stimulator. The signal from the punctate stimulation was passed to a field pulse controller that differentiated the signal and triggered a square wave pulse. This pulse, when allowed, triggered the start of a train of electrical field pulses, delivered *via* a Grass S88C dual stimulator to a pair of platinum wire field electrodes that were positioned parallel to the atrium. This equipment could deliver field pulses across the tissue in the atrial refractory period (40–60 ms long) to avoid conduction disturbances but allow depolarization of the autonomic varicosities and the release of neurotransmitters (Angus & Harvey, 1981). This method elicited graded changes in atrial force that were linear with respect to the number of applied field pulses. The signal from both channels of the S88C were monitored on a dual beam 10 MHz storage oscilloscope. The signal from the force transducer was amplified and atrial force of contraction continuously recorded on a chart recorder (Neotrace 600ZF).

Protocol

Effect of calcium channel blockers on inotropic responses to EFS To assess responses to vagal stimulation, atria were washed frequently for 30 min in Krebs' containing 1 μM propranolol and 1 μM clonidine (Serone & Angus, 1999). To assess responses to sympathetic stimulation, atria were washed frequently for 30 min in Krebs' containing 1 μM atropine. At the end of this equilibration period the response to electrical field stimulation (EFS) was assessed (as above) by applying four field pulses per refractory period (0.1 ms duration, 200 Hz, 100 V on S88 dial) following the driving (punctate) pulse for eight consecutive pulses (Serone & Angus, 1999). The subsequent changes in atrial force (g) were measured. A second control stimulus (C2) was performed 15 min after the initial response to EFS to test the reproducibility of the inotropic response. Cumulative concentration-response curves were then constructed to ω-conotoxin GVIA (0.1–10 nM) or ω-conotoxin MVIIC (0.01–1 μM). ω-conotoxin GVIA was incubated for 60 min at each concentration. ω-conotoxin MVIIC was incubated for 60 min (sympathetic responses) or 30 min (vagal responses) per concentration. The inotropic response to EFS was reassessed at the end of each equilibration time for each concentration of toxin. Time control experiments were also performed for both vagal and sympathetic experiments.

Drugs

Drugs used were freshly prepared in ultra-filtered water (Milli Q UV) and included atropine sulphate (Sigma, St Louis, MO, U.S.A.), clonidine hydrochloride (Boehringer, Ingelheim), ω-conotoxin MVIIC (Peptide Institute Inc., Osaka, Japan) and propranolol hydrochloride (Sigma). ω-conotoxin GVIA (synthesized by Dr Roger Murphy, Department of Pharmacology, University of Melbourne, Victoria, Australia). Homogeneity of the peptide was confirmed by analytical HPLC and capillary electrophoresis (Lew *et al*., 1997).

Analysis and statistical methods

Parameter measurement and agonist concentration-response curves Data are presented as mean $\pm$ 1 standard error of the mean (s.e.mean). The decrease in force of contraction of the atria (V_1 vagal response) following EFS was calculated by subtracting the force of contraction (in g) at the peak of the negative inotropic response to EFS (V_2 from the force of contraction at baseline immediately prior to EFS (*b*). The decrease in force was then expressed as per cent inhibition of baseline contraction (see Figure 1). The sympathetically-mediated inotropic response to EFS (*S*) was measured as the peak increase in contractile force of the atria following EFS (in g). The positive inotropic response to EFS was then expressed as per cent increase in atrial force (see Figure 1). Individual peptide concentration-response curves were fitted to a sigmoid logistic equation:

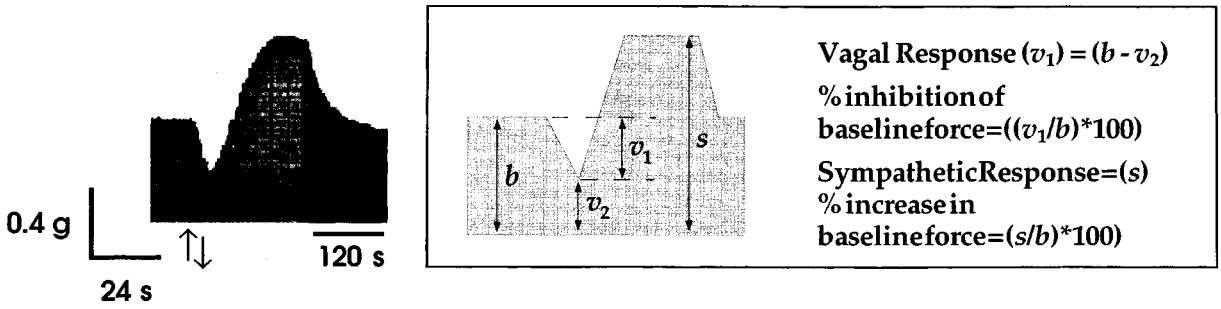

Figure 1 Representative trace recording of the inotropic response to eight trains electrical field stimulation (EFS; each train was four pulses per refractory period, see Methods) in a guinea-pig isolated left atrium in the absence of antagonists. EFS was passed across the atria as indicated by the upward and downward arrows. Second calibration bar indicates slowing of the chart speed from 25 to 5 mm min^{-1}; Insert shows a schematic representation of the analysis of the inotropic responses to EFS (see Methods). b is the baseline force of contraction immediately prior to EFS. v_1 is the vagally-mediated decrease in punctate force of contraction, calculated as the difference between b and the peak force of contraction at the end of EFS (v_2). The sympathetically-mediated positive inotropic response to EFS is measured as the difference between the maximum increase in the force of contraction following EFS (s) and b.

$$\text{Response} = \frac{a+b}{1+e^{d(c+x)}} \quad (1)$$

where a is the resting level of response, b is the response range, c is the $-\log_{10}$ of the molar concentration that elicits 50% of the maximum response (pEC_{50}), d is the slope and curvature parameter and e is the base of the natural logarithm (Lew & Angus, 1995). To compare the effects of GVIA and MVIIC on sympathetic and vagal inotropic responses, data were plotted on a common y-axis by expressing the effect of the conotoxins on the inotropic response to EFS as a percentage of C2. Changes in basal contractile force with time were compared between treatment groups by repeated measures ANOVA. The Greenhouse-Geisser estimate of epsilon was used as a correction for correlation (Ludbrook, 1994). In control atria, the negative or positive inotropic response to eight trains EFS with time was compared by two-way ANOVA. C2 responses were compared between treatment groups by one-way ANOVA. The potency (pIC_{50}) of GVIA or MVIIC at inhibiting the vagal and sympathetic inotropic responses to EFS were compared by Students' t-test for unpaired data.

In all cases, statistical significance was accepted when $P<0.05$.

Results

Basal contractility of left atria: effect of time and ω-toxins

Initial force of contraction was not different among experimental groups therefore the data was pooled. Average force of contraction of the atria immediately prior to the first control stimulus was 0.36 ± 0.03 g, range 0.14–0.88 g ($n=30$).

In the experiments examining sympathetic neural responses, there was a trend for basal contractile force to fade over time in all three experimental groups pretreated with atropine (1 μM). However, this time dependent fade in basal contractile strength was not significant after correction for repeated measures ($P=0.1142$, $n=15$).

In experiments examining vagal stimulation, atria pretreated with propranolol (1 μM) and clonidine (1 μM) showed a significant decline in the basal force of contraction with time ($P=0.003$, $n=15$). However, there no was significant difference in the rate of fade of basal contractility between toxin treatment groups and the corresponding time controls ($P=0.1161$).

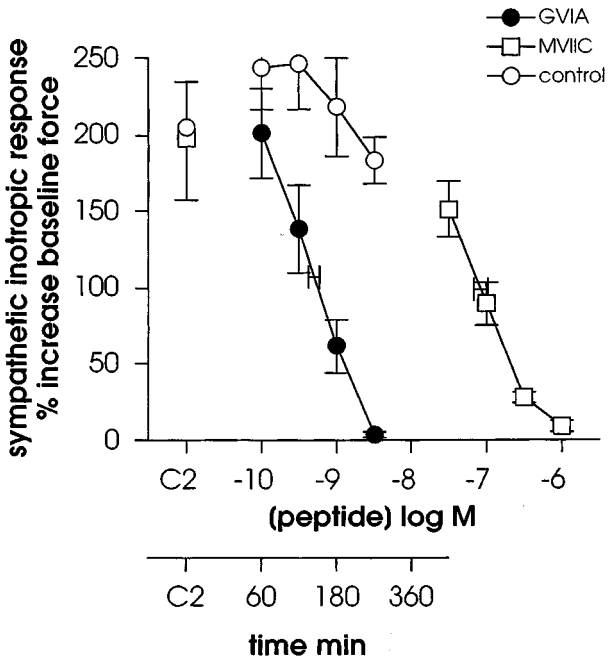

Figure 2 Effect of calcium channel blockers on the sympathetic response to field stimulation (EFS) of guinea-pig isolated left atria in the presence of atropine (1 μM). Curves are the positive inotropic response to eight trains EFS (each train was four pulses per refractory period; see Methods) expressed as per cent increase in baseline force (see Methods) following cumulative additions of either ω-conotoxin GVIA (GVIA; 0.1–3 nM; $n=5$) or ω-conotoxin MVIIC (MVIIC; 30–1000 nM; $n=5$). Each concentration of peptide was incubated for 60 min prior to assessing the response to EFS. The positive inotropic response to EFS with time in control atria ($n=5$) is shown for comparison. Vertical error bars are $\pm$s.e.mean. Horizontal error bars are ± 1 s.e.mean on the average fitted IC_{50} for GVIA and MVIIC. C2 is the second control inotropic response to EFS immediately prior to addition of the first concentration of peptide or vehicle.

Effect of calcium channel blockers on the positive inotropic response to EFS

There was no significant difference in the second control positive inotropic response (C2) to eight trains electrical field stimulation (EFS) between experimental groups (Figure 2). Incubation of the atria with the selective N-type calcium channel blocker ω-CTX GVIA (GVIA; 0.1–3 nM) caused a

concentration dependent inhibition of the sympathetically-mediated positive inotropic response to EFS in the presence of atropine (1 μM) (Figure 2, pIC_{50} GVIA, 9.29 ± 0.08). ω-CTX MVIIC (MVIIC), a calcium channel blocker that inhibits both N-type and P/Q type calcium channels, also decreased the positive inotropic response to EFS with approximately 160 fold lower potency than ω-CTX GVIA (Figure 2, pIC_{50} MVIIC, 7.08 ± 0.10 M). There was no significant effect of time on the sympathetic response to EFS in control atria (Figure 2). Figure 3 shows a representative experimental chart record of the effect of ω-conotoxin GVIA on the positive inotropic response to eight trains EFS.

Effect of calcium channel blockers on the negative inotropic response to EFS

There was no significant difference in the control negative inotropic response (C2) to eight trains EFS between experimental groups (Figure 4). Both ω-conotoxins GVIA (0.3 – 10 nM) and MVIIC (10 – 1000 nM) caused a concentration-dependent inhibition of the vagally-mediated negative inotropic response to EFS (Figures 3 and 4). However, GVIA (0.1 – 3 nM) was 85 times more potent at inhibiting the negative inotropic response to EFS than MVIIC (9.13 ± 0.17 and 7.20 ± 0.14; pIC_{50} GVIA and MVIIC, respectively). There was no effect of time on the negative inotropic response to EFS in control atria (Figure 4).

Figure 5 shows a comparison of the effects of ω-conotoxins on the sympathetic and vagal response to EFS. The data was plotted on a common axis by expressing the effects of each concentration of toxin on the inotropic responses to EFS as a percentage on the second control stimulus (C2). It is immediately apparent from this data that the sensitivity of both GVIA and MVIIC are independent of the inotropic response being studied i.e. both toxins are equipotent at eliciting both the negative and positive inotropic responses to EFS.

Discussion

These data demonstrate that blockade of N-type calcium channels caused complete inhibition of cardiac autonomic neurotransmission in the guinea-pig isolated left atrium. The highly selective N-type calcium channel blocker, ω-conotoxin GVIA (GVIA) was equipotent at inhibiting both vagal and sympathetic inotropic responses to field stimulation. Likewise, the calcium channel blocker ω-conotoxin MVIIC (MVIIC; non-selective between N-type and P/Q-type at concentrations >1 nM (Hillyard *et al.*, 1992)) was also equally effective at inhibiting both vagal and sympathetic inotropic responses. However, MVIIC was between 85 – 160 fold less potent than GVIA at inhibiting inotropic responses.

Depolarization of the cardiac myocyte membrane – either *via* propagation of pacemaker action potential or direct depolarization of the muscle with electrical field pulses – and subsequent opening of L-type calcium channels is essential for the influx of extracellular calcium required for myocyte contraction (Godfraind *et al.*, 1986). Incubation of the atria with increasing concentrations of either ω-conotoxin GVIA or ω-conotoxin MVIIC had no effect on basal contractility of the left atria, consistent with previous findings that the toxins used

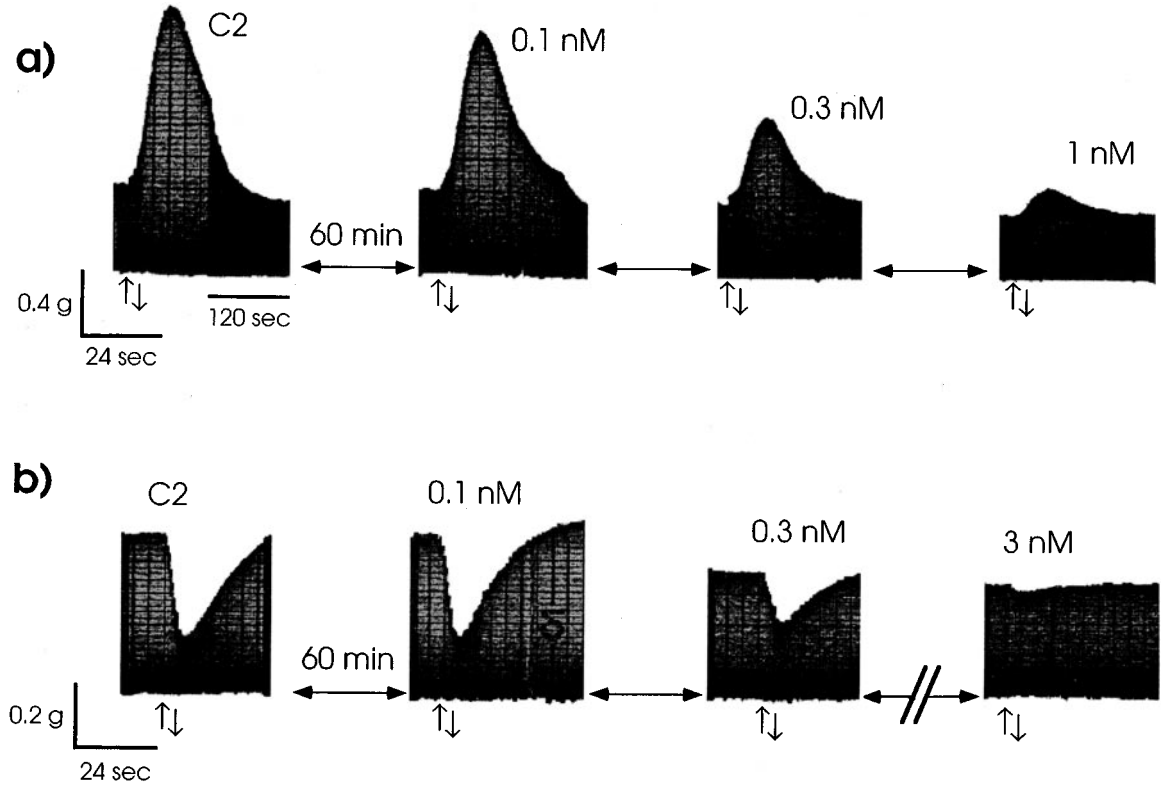

Figure 3 Representative experimental chart record of the effect of ω-conotoxin GVIA (GVIA) on inotropic responses to eight trains electrical field stimulation (EFS). EFS was passed across the atria as indicated by the upward and downward arrows. (a) is the effect of GVIA on the sympathetic nerve-mediated positive inotropic response to EFS in the presence of atropine (1 μM). Second calibration bar on (a) indicates slowing of the chart speed from 25 to 5 mm min^{-1}. (b) is the effect of GVIA on the vagal nerve mediated negative inotropic response to EFS in the presence of propranolol (1 μM) and clonidine (1 μM).

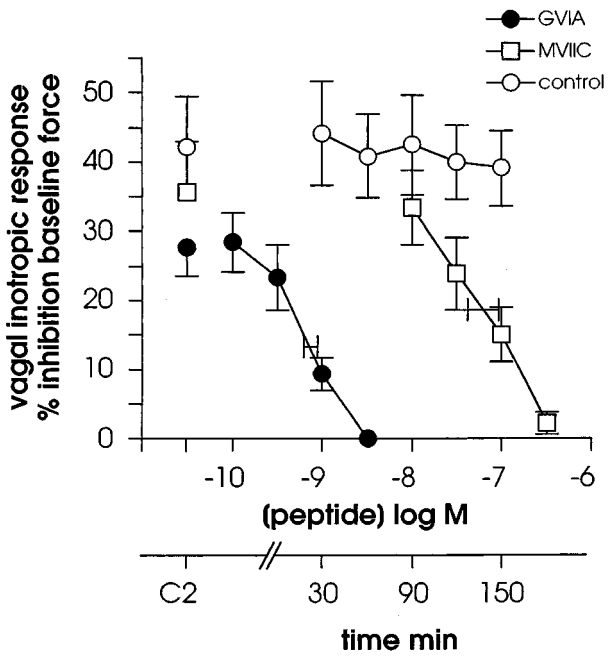

Figure 4 Effect of calcium channel blockers on the vagal response to field stimulation (EFS) of guinea-pig isolated left atria in the presence of propranolol (1 μm) and clonidine (1 μM). Curves are the negative inotropic response to eight trains EFS (each train was four pulses per refractory period; see Methods) expressed as per cent inhibition of baseline force (see Methods) following cumulative additions of either ω-conotoxin GVIA (GVIA; 0.1 – 3 nM; $n=5$) or ω-conotoxin MVIIC (10 – 300 nM; $n=5$). Each concentration of peptide was incubated for either 60 min (GVIA) or 30 min (MVIIC) prior to assessing the response to EFS. The negative inotropic response to EFS with time in control atria ($n=5$) is shown for comparison. Vertical error bars are $\pm$s.e.mean. Horizontal error bars are $\pm$1 s.e.mean on the average fitted IC_{50} for each curve. C2 is the second control inotropic response to EFS immediately prior to addition of the first concentration of peptide or vehicle.

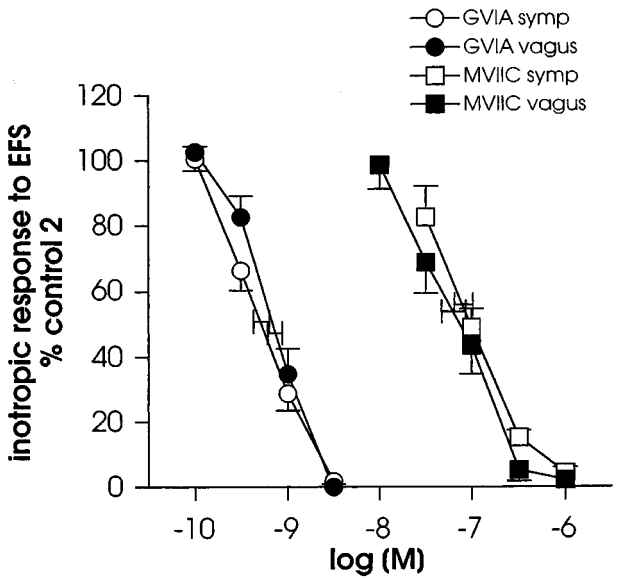

Figure 5 Effect of calcium channel blockade by ω-conotoxins on the positive and negative inotropic responses to field stimulation in guinea-pig isolated left atria. Curves are the inotropic response to eight trains EFS (each train was four pulses per refractory period; see Methods) expressed as per cent of the second control response to 8 trains EFS (see Methods) immediately prior to addition of cumulative concentrations of either ω-conotoxin GVIA (GVIA; 0.1 – 10 nM; $n=5$) or ω-conotoxin MVIIC (10 – 1000 nM; $n=5$). GVIA was incubated for 60 min at each concentration. MVIIC was incubated for 60 min (sympathetic responses) or 30 min (vagal responses) at each concentration. Vertical error bars are $\pm$s.e.mean. Horizontal error bars are $\pm$1 s.e.mean on the average fitted IC_{50} for each curve.

in this study are not active at the L-type calcium channel (Olivera *et al.*, 1994).

N-type calcium channels mediating sympathetic neurotransmission

The N-type calcium channel appears to be the principle calcium channel involved in neurosecretion in most peripheral, sympathetically-innervated tissues reported to date (Olivera *et al.*, 1994). In isolated cardiac muscle preparations, GVIA causes potent inhibition of sympathetically-mediated increases in atrial rate (De Luca *et al.*, 1990; Ren *et al.*, 1993; Wright & Angus, 1995; 1996) or force (Hong & Chang, 1995; Vega *et al.*, 1995) in response to field stimulation. Furthermore, GVIA administered intravenously into intact animal preparations causes marked sympatholytic effects (Pruneau & Angus, 1990a,b; Wright & Angus, 1995). Previously, Hong & Chang (1995) demonstrated that GVIA and MVIIC caused inhibition of the positive inotropic response to EFS in the guinea-pig left atria with equal potency (6.4 & 6.3, pIC_{50} GVIA & MVIIC, respectively). Whilst the potency of MVIIC at inhibiting the sympathetically-mediated increase in force reported by Hong & Chang (1995) was similar to the results presented here (pIC_{50} MVIIC, 7.08 ± 0.10), we found GVIA to be almost three orders of magnitude more potent by comparison (pIC_{50} 9.29 ± 0.08). Inhibition of the sympathetic inotropic response to field stimulation with nanomolar concentrations of GVIA demonstrated here is consistent with the reported potency of GVIA causing inhibition of N-type calcium current and the subsequent release of noradrenaline in superior cervical ganglion cells (Hirning *et al.*, 1988). Furthermore, the finding that GVIA was about 160 times more potent than MVIIC at inhibiting sympathetic responses is consistent with previously reported differences in the binding affinities of the two toxins at the high affinity GVIA binding site in rat brain membranes (N-type calcium channel) (Hillyard *et al.*, 1992). Others have also reported GVIA has a potency between 7 – 100 fold greater than MVIIC in a variety of *in vitro* preparations (Vega *et al.*, 1995; Hong *et al.*, 1996; Waterman, 1996; 1997; Wright & Angus, 1996). A recent study by Vega *et al.*, (1995) demonstrated GVIA inhibited sympathetically-mediated inotropic responses to field stimulation of the guinea-pig isolated left atrium with a potency similar to that reported by Hong & Chang, (1995). GVIA has previously been shown to be selective for the N-type calcium channel, even at concentrations four orders of magnitude greater than those required to inhibit sympathetic nerve-mediated responses in the isolated small mesenteric artery (Whorlow *et al.*, 1996). In contrast, in non-cardiovascular experimental systems, micromolar concentrations of GVIA have previously been shown to cause *reversible* blockade of non-N-type neuronal calcium channels (Williams *et al.*, 1992; Ellinor *et al.*, 1993). This may suggest that the GVIA-mediated inhibition reported by Hong & Chang (1995) and Vega *et al.* (1995) may have been due to blockade of these other non-N-type calcium channels at the concentrations used by these investigators. However, both Hong & Chang (1995) and Vega *et al.* (1995) demonstrated the blockade elicited by GVIA to be irreversible, suggesting that inhibition of cardiac inotropic responses by blockade of other, non-N-type calcium

channels at these concentrations of GVIA would appear unlikely. The most likely explanation for the difference in the potency of GVIA between the previous studies of Vega *et al.* (1995) and Hong & Chang (1995) and the results presented here, is the difference in incubation time allowed before assessing the effects of each toxin on nerve-mediated inotropic responses. Both Hong & Chang (1995) and Vega *et al.* (1995) incubated their toxins for periods of between 10–20 min before assessing responses. GVIA has previously been shown to have an extremely slow onset of action on cardiac sympathetic nerves in both rat and guinea-pig isolated right atria (Angus, J.A., unpublished observations). When the time course of inhibition in response to a low concentration of GVIA is examined in both these tissues, distinct differences in time to equilibration can be observed. GVIA (1 nM) shifts the relationship between sympathetically-mediated tachycardia and electrical field pulse stimulation to the right, curves not becoming stationary until 60 min (rat) or 120 min (guinea-pig) after incubation (Angus, J.A. unpublished observations). Similar results demonstrating the slow onset of GVIA compared to MVIIC have been reported by Boot (1994). The time dependent nature of GVIA-mediated inhibition of the rat isolated vas deferens preparation has also been previously demonstrated by Maggi *et al.* (1988). In this study, an incubation time of 60 min was chosen to study the effects of GVIA on autonomic neurotransmission in the guinea-pig atrium. Thus, the extremely slow time course for onset of low concentrations of GVIA would imply that even with 60 min incubation time, the potency of GVIA is actually being greatly underestimated.

N-type calcium channels mediating vagal neurotransmission

Vagally-mediated negative inotropic responses to field stimulation of the guinea-pig isolated left atrium were recently shown to be insensitive to the effects of GVIA (Hong & Chang, 1995). However, as demonstrated in the current study, GVIA caused potent (pIC_{50} 9.13 ± 0.17) and complete inhibition of vagal neurosecretion in the guinea-pig isolated left atrium. In contrast to the reported effects of GVIA, MVIIC was previously shown to mediate complete inhibition of the negative inotropic response to field stimulation (pIC_{50} 6.55, Hong & Chang, 1995), This effect of MVIIC is consistent with our findings for vagal inhibition in the current study, albeit with a slightly lower potency than demonstrated here (pIC_{50} 7.20 ± 0.14). Evidence for N-type calcium channel involvement in cholinergic autonomic neurotransmission is based primarily on studies examining the effects of GVIA and other calcium channel blockers on nerve-mediated responses of isolated gastrointestinal or urogenital preparations. Parasympathetically-mediated contractions of the ileum are consistently inhibited by GVIA in a variety of species (Lundy & Frew, 1988; 1994; De Luca *et al.*, 1990; Boot, 1994; Hong *et al.*, 1996). However, saturating concentrations of GVIA inhibit field stimulation-induced contractions by only 40% in the rat isolated detrussor muscle (Frew & Lundy, 1995). It is worth noting that significant resistance to atropine was demonstrated in this tissue (80% with 1 μM) suggesting that nerve-mediated responses were elicited by a combination of cholinergic and non-adrenergic, non-cholinergic (NANC) neurotransmission and that GVIA may therefore also affect NANC responses (Frew & Lundy, 1995). Likewise, in the mouse urinary bladder-dome preparation (detrussor muscle), N-type calcium channels appear to be selectively coupled to the release of acetylcholine from postganglionic parasympathetic nerves (Waterman, 1996), although a significant proportion of the contractile response to nerve stimulation (60%) was resistant to increasing concentrations of GVIA (to 1 μM, Waterman, 1996). Resistance of cholinergic neurotransmission to the blocking effects of GVIA have also been reported for the cardiac vagus. Intravenously administered GVIA (3 $\mu g\ kg^{-1}$) causes profound sympatholytic effects with no apparent inhibition of the efferent cardiac vagus, as demonstrated by no change in the maximum decrease in heart-rate elicited by activation of cardiac autonomic reflexes (Pruneau & Angus, 1990b). Likewise, in the spontaneously beating rabbit isolated right atrium, 1 nM GVIA has no effect on vagally-mediated increases in atrial period (Wright & Angus, 1995). However, it has since been demonstrated that higher concentrations of GVIA are able to effectively inhibit vagal responses in the rabbit isolated right atrium, near 100% inhibition occurring at 100 nM (Wright & Angus, unpublished observations) confirming the existence of N-type calcium channels on cardiac vagal efferents in this species. The low potency of GVIA demonstrated in the rabbit isolated right atrium may explain why GVIA appears to be a selective inhibitor of sympathetic neurotransmission when administered into the conscious animal. However, the low potency of GVIA on the cardiac vagus appears to be species dependent. Ren *et al.* (1993) previously demonstrated 3 nmol GVIA causes near complete inhibition of the vagal chronotropic response to electrical stimulation of the dog isolated right atrium, a finding in keeping with the effects of GVIA on vagal responses in the current study.

One theory that can be proposed for the apparent difference in the potency of GVIA at inhibiting autonomic neurotransmission between species is the 'spare channel' theory put forward by Dunlap *et al.* (1994). This theory is analogous to the situation of spare receptors for a full agonist with high intrinsic efficacy. However, the 'spare channel' theory has been disputed by Wheeler *et al.* (1994) who suggest that calcium entry and neurosecretion obey a classical power-law relationship and that under these circumstances saturation of calcium during neurosecretion is unlikely to occur. Wheeler *et al.* (1994) do concede that 'spare channels' may exist when prolonged and strong depolarizations are used, such that the likelihood of an individual calcium channel conducting enough calcium to trigger neurosecretion is increased. The duration of the field pulses in this study was only 0.1 ms, in contrast, the study examining the effects of GVIA in the rabbit isolated right atrium utilized field pulses of 2 ms duration (Wright & Angus, 1995). Therefore, although there is controversy regarding the relevance of the 'spare channel' theory, it is interesting to speculate that this situation may exist between species or that experimental conditions must be taken into account when examining the ability of calcium channel antagonists to inhibition neurotransmission. However, this situation does not resolve the apparent difference in GVIA potency between the report of Hong & Chang (1995) and the findings of this study, as the same preparation from the same species was used to examine the effects of the ω-conotoxins on peripheral autonomic neurotransmission. These discrepancies are inexplicable.

In conclusion, these data demonstrate that calcium entry *via* the N-type calcium channel accounts for all of the calcium current required for cardiac autonomic neurotransmission in the guinea-pig isolated left atrium. The presence of N-type calcium channels on both efferent components of the autonomic nervous system in the heart is consistent with the ability of NPY to mediate inhibition of cardiac vagal and sympathetic neurotransmission *via* closure of these channels.

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