British Journal of Pharmacology (2004) 141, 961–970
www.nature.com/bjp

Differential involvement of N-type calcium channels in transmitter release from vasoconstrictor and vasodilator neurons

*,[1]Judy L. Morris, [1]Daina I. Ozols, [2]Richard J. Lewis, [1]Ian L. Gibbins & [1]Phillip Jobling

[1]Department of Anatomy & Histology, Centre for Neuroscience, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia and [2]Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD 4072, Australia

1 The effects of calcium channel blockers on co-transmission from different populations of autonomic vasomotor neurons were studied on isolated segments of uterine artery and vena cava from guinea-pigs.

2 Sympathetic, noradrenergic contractions of the uterine artery (produced by 200 pulses at 1 or 10 Hz; 600 pulses at 20 Hz) were abolished by the N-type calcium channel blocker ω-conotoxin (CTX) GVIA at 1–10 nM.

3 Biphasic sympathetic contractions of the vena cava (600 pulses at 20 Hz) mediated by noradrenaline and neuropeptide Y were abolished by 10 nM CTX GVIA.

4 Neurogenic relaxations of the uterine artery (200 pulses at 10 Hz) mediated by neuronal nitric oxide and neuropeptides were reduced $<50\%$ by CTX GVIA 10–100 nM.

5 Capsaicin (3 μM) did not affect the CTX GVIA-sensitive or CTX GVIA-resistant neurogenic relaxations of the uterine artery.

6 The novel N-type blocker CTX CVID (100–300 nM), P/Q-type blockers agatoxin IVA (10–100 nM) or CTX CVIB (100 nM), the L-type blocker nifedipine (10 μM) or the 'R-type' blocker SNX-482 (100 nM), all failed to reduce CTX GVIA-resistant relaxations. The T-type channel blocker $NiCl_2$ (100–300 μM) reduced but did not abolish the remaining neurogenic dilations.

7 Release of different neurotransmitters from the same autonomic vasomotor axon depends on similar subtypes of calcium channels. N-type channels are responsible for transmitter release from vasoconstrictor neurons innervating a muscular artery and capacitance vein, but only partly mediate release of nitric oxide and neuropeptides from pelvic vasodilator neurons.
British Journal of Pharmacology (2004) **141,** 961–970. doi:10.1038/sj.bjp.0705712

Keywords: Co-transmission; ω-conotoxin; voltage-gated calcium channels; pelvic neurons; vasodilation; sympathetic; vasoconstriction; neuropeptides; nitric oxide; capsaicin

Abbreviations: ACh, acetylcholine; ANCOVA, analysis of covariance; ANOVA, analysis of variance; ATX, agatoxin IVA; BoNTA, botulinum neurotoxin A; CTX, conotoxin; HEPES, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid; L-NAME, N^G-nitro-L-arginine methyl ester; NA, noradrenaline; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; SNAP-25, synaptosomal associated protein of 25 kDa; SNARE, soluble NSF attachment protein receptor; VIP, vasoactive intestinal peptide

Introduction

Neural regulation of vascular resistance and capacitance utilises multiple vasoconstrictor and vasodilator pathways projecting from the central nervous system to precise vascular targets (Gibbins & Morris, 2000). Most sympathetic vasoconstrictor neurons contain the co-transmitters noradrenaline (NA), ATP and neuropeptide Y (NPY). Vasodilator neurons in cranial and pelvic autonomic pathways utilise nitric oxide (NO), acetylcholine (ACh) and neuropeptides such as vasoactive intestinal peptide (VIP). The co-transmitters contributing to autonomic vasoconstriction or vasodilation can vary considerably between vascular beds and with different patterns of stimulation (Gibbins & Morris, 2000). The latter variability can be at least partly attributed to the release of co-transmitters from different intracellular compartments (Lundberg & Hökfelt, 1986).

In neurons innervating vascular smooth muscle, exocytosis of transmitter in response to single pulses and low levels of repetitive stimulation occurs predominantly from small synaptic vesicles, whereas transmitter release from large vesicles generally requires more prolonged periods of stimulation or higher pulse frequency. ATP and NA are released from small and large synaptic vesicles in sympathetic vasoconstrictor nerves, whereas NPY is released only from large vesicles (Lundberg & Hökfelt, 1986). NO-mediated autonomic vasodilation occurs after NO synthesis from arginine in the neuronal cytoplasm (Bredt & Snyder, 1990), and often reaches a maximal effect at lower stimulation frequencies. In contrast, slower vasodilator responses at higher stimulation frequencies are produced by neuropeptides released from large vesicles (Lundberg & Hökfelt, 1986). ACh is released from small and large synaptic vesicles, but does not always contribute directly to neurogenic vasodilation in cranial and pelvic vessels (Morris, 1993).

*Author for correspondence: E-mail: Judy.Morris@flinders.edu.au
Advance online publication: 1 March 2004

A key event in exocytosis of neurotransmitters from synaptic vesicles is the entry of calcium through voltage-gated calcium channels in the nerve terminal, and interaction of calcium with soluble NSF attachment protein receptor (SNARE) proteins on the vesicle and nerve terminal membranes (Söllner *et al*., 1993). Indeed, there is a direct interaction between calcium channel α_1 subunits and the SNARE proteins synaptotagmin, syntaxin and synaptosomal associated protein of 25 kDa (SNAP-25) during docking and fusion of synaptic vesicles (Sheng *et al*., 1988). Synthesis of NO by the calmodulin-dependent enzyme neuronal nitric oxide synthase (nNOS) also is stimulated by increases in intracellular calcium (Bredt, 1999).

Voltage-gated calcium channels mediating transmitter release from autonomic neurons commonly are N- or P/Q-type (Waterman, 2000). Some autonomic neurons also utilise calcium channels resistant to N- or P/Q-type channel blockers (Smith & Cunnane, 1997). There is evidence that release of co-transmitters can be mediated by entry of calcium through different channels into the same autonomic neuron (Waterman, 1996). In other cases, release of co-transmitters seems to involve the same channel subtypes (Waterman, 1997; Brock & Cunnane, 1999; Yang & Chiba, 2000).

Transmitter release from different populations of autonomic vasomotor neurons is differentially sensitive to botulinum neurotoxin A (BoNTA) cleavage of the SNARE protein, SNAP-25 (Morris *et al*., 2001; 2002). Furthermore, co-transmitters released from the same vasomotor neurons are not equally sensitive to BoNTA. Vasodilation of the uterine artery mediated by neuronal NO is insensitive to BoNTA, peptide-mediated dilation is partly reduced, and cholinergic pre-synaptic inhibition is abolished by BoNTA (Morris *et al*., 2001). Release of NA from vasoconstrictor neurons in the uterine artery and vena cava is partly reduced by BoNTA, but NPY-mediated slow constriction of the vena cava is not affected by BoNTA (Morris *et al*., 2001; 2002). These results suggest that the role of SNARE proteins in transmitter release differs between co-transmitters and between populations of vasomotor neurons. A possible contributing factor is the involvement of different subtypes of calcium channels in neurotransmitter release.

This study set out to test the hypotheses that: (1) different subtypes of voltage-gated calcium channels are involved in release of co-transmitters from the same vasomotor neurons; (2) different channel subtypes are involved in transmitter release from different populations of vasomotor neurons. We have examined the role of voltage-gated calcium channels in transmission from sympathetic vasoconstrictor neurons to the uterine artery and inferior vena cava of guinea-pigs, and from pelvic vasodilator neurons supplying the uterine artery, using blockers of N-, P/Q-, L-, T- or R-type calcium channels.

Methods

Guinea-pigs (Hartley-IMVS, 250–350 g body weight) were killed by stunning and exsanguination *via* the carotid arteries, prior to removal of the thoracic portion of the inferior vena cava or the main uterine artery. These experiments were approved by the Flinders University Animal Welfare Committee, in accordance with the guidelines of the National Health and Medical Research Council of Australia.

In vitro *preparations*

The thoracic portion of the inferior vena cava or the main uterine artery was removed and placed in HEPES-buffered balanced salt solution (composition in mM: 146 NaCl, 4.7 KCl, 0.6 $MgSO_4$, 1.6 $NaHCO_3$, 0.13 NaH_2PO_4, 2.5 $CaCl_2$, 7.8 glucose, 20 HEPES, 0.1 ascorbic acid) adjusted to pH 7.3 with NaOH and bubbled with 100% O_2 at 36°C. Venae cavae were mounted in a 10 ml organ bath on two parallel wires and were stretched until the equivalent of 10 mN force was applied. A ring segment 4–5 mm long was dissected from the caudal end of the uterine artery and stretched between two nichrome wires (25 μm diameter) until the wires were separated by 1–1.25 times the resting internal circumference. The endothelium is intact in these preparations. Arteries were lowered into a 1 ml bath and were superfused with HEPES-buffered solution. All vessels were left to equilibrate for 1 h.

Perivascular axons were stimulated *via* two platinum wires positioned on either side of and parallel to the long axis of the vessel. Trains of pulses of 0.3 ms duration were delivered by a Grass S44 stimulator connected to a low impedance interface (Biomedical Engineering, Flinders Medical Centre). Changes in isometric force were detected with a Grass FT03 transducer (venae cavae) or a Gould-Statham UTC2 transducer (uterine artery) and recorded digitally using Chart v4.0 or v4.2 on a PowerLab 4S (AD Instruments, Castle Hill, NSW, Australia) connected to a Power Macintosh 7600/200 (Apple Computer Inc., CA, U.S.A.).

For the vena cava, changes in force produced by field stimulation of perivascular sympathetic axons were calibrated in mN. The magnitude of responses, taking into account the amplitude and duration, was defined as the area under the contractile response. The magnitude was measured using Chart v4.2 and NIH Image v1.62. The time from the start of the stimulation period to the initial rise in force (latency) also was determined. An increase in latency has been shown to be a good index of blockade of the initial noradrenergic component of the sympathetic response, while reduction in the area is a good index of blockade of the NPY component (Morris *et al*., 2002). Contractions produced by 126 mM KCl were measured at the end of each experiment.

Sympathetic contractions of the uterine artery were calibrated in mN. Under the conditions of these experiments, the sympathetic contractions at 1, 10 and 20 Hz are mediated by noradrenaline acting on α_1-adrenoceptors (Morris *et al*., 2002).

Neurogenic relaxations of the uterine artery were examined after blockade of neurotransmission from vasoconstrictor neurons with guanethidine (1 μM) and precontraction with phenylephrine (0.3 μM) to approximately 50% of the maximal contraction produced by KCl at the conclusion of each experiment. Data were used only from those experiments where the initial contraction produced by phenylephrine was between 35 and 65% of the KCl contraction, and the level of contraction decreased by less than 50% during the experiment. Under these conditions, neurogenic dilations were always endothelium-independent and biphasic, the first phase mediated by neuronal nitric oxide (Morris, 1993; Morris *et al*., 2001) and the second phase mediated by the neuropeptides VIP and/or CGRP (Morris, 1993; Anderson *et al*., 1997). The magnitude of the first, nitrergic component of neurogenic dilations was determined by expressing the fall in tension during the stimulation period as a percentage of the

level of precontraction above baseline. As an index of magnitude of the second, peptide-mediated phase of vasodilation, the integrated response was calculated as the area of the relaxation below the level of precontraction as a percentage of the total area down to baseline (Morris *et al*., 2001). Control experiments with repeated periods of stimulation in the absence of blocking agents have confirmed that these normalised values provide reliable indices of the magnitude of vasodilations over time, even in those preparations with a slightly falling 'baseline' of precontraction.

Calcium channel blockade

Protocol 1: effect of the N-type channel blocker ω-conotoxin GVIA on sympathetic constrictions of the vena cava. Eight veins were stimulated with trains of 600 pulses delivered at 20 Hz every 20 min in the presence of propranolol (0.3 μM) to block β-adrenoceptors (see Morris, 1991). After three control stimuli, ω-conotoxin (CTX) GVIA was applied to the organ bath at 1 nM ($n=4$) or 10 nM ($n=4$) and was present for a further four to seven stimulation periods.

Protocol 2: effect of the N-type channel blocker ω-conotoxin GVIA on sympathetic constrictions of the uterine artery. 2a: Arteries from five animals were stimulated every 20 min with trains of 200 pulses, with pulse frequencies of 1 or 10 Hz in alternate stimulation periods. CTX GVIA at 1 nM was added to the superfusate after two control stimuli at each frequency, and was present for a further two stimuli at each frequency. 2b: Arteries were stimulated every 30 min with trains of 600 pulses at 20 Hz. Segments from four animals were treated with 1 nM CTX GVIA after the third of 10 stimulation periods. Arteries from further five animals were treated with 10 nM CTX GVIA after three control stimulation periods.

Protocol 3: dose–response effect of the N-type channel blocker ω-conotoxin GVIA on neurogenic dilations of the uterine artery. Arteries from 15 animals were precontracted with phenylephrine (0.3 μM). in the presence of guanethidine (1 μM) and were stimulated every 20 min with trains of 200 pulses at 10 Hz. CTX GVIA at 1 nM ($n=5$), 10 nM ($n=5$) or 30 nM ($n=5$) was added to the superfusate after the second of five or six stimulation periods.

Protocol 4: effect of ω-conotoxin GVIA on neurogenic dilations of the uterine artery after capsaicin pretreatment in vitro. In preliminary experiments ($n=3$), neurogenic relaxations to trains of 200 pulses at 10 Hz were obtained in arteries pretreated with phenylephrine and guanethidine, then capsaicin (1–3 μM) was applied for 6 min and washed out. After four to six more stimulation periods, capsaicin was reapplied for 6 min. In separate experiments ($n=4$), capsaicin (3 μM) was added to the superfusate after 50 min of the equilibration period, then washed out after 6 min, prior to addition of guanethidine and precontraction with phenylephrine. Six trains of 200 pulses at 10 Hz were applied at 30 min intervals. CTX GVIA at 30 nM was added to the superfusate between the second and third stimuli, then nitro-L-arginine methylester (L-NAME; 10 μM) was added between the fourth and fifth stimulation periods. CTX GVIA and L-NAME were then washed out, and capsaicin (3 μM) was reapplied for 6 min. A further four arteries were pretreated with ethanol/Tween 80 vehicle instead of capsaicin, prior to CTX GVIA exposure.

Protocol 5: effect of the P/Q-type channel blocker ω-agatoxin IVA on neurogenic dilations of the uterine artery. Five arteries precontracted with phenylephrine in the presence of guanethidine (1 μM) were stimulated four times with trains of 200 pulses at 10 Hz at 20 min intervals. Agatoxin (ATX) IVA at 10 nM was added to the superfusate between the second and third stimulation periods. In a further five experiments, CTX GVIA (100 nM) was applied to recirculating superfusate between the second and third stimuli; then ATX at 100 nM was added to the CTX GVIA between the fourth and fifth stimulation periods.

Protocol 6: effects of the N/P/Q-type channel blocker, ω-conotoxin CVIB, and the novel N-type channel blocker, ω-conotoxin CVID, on neurogenic dilations of the uterine artery. Arteries from five animals were precontracted with phenylephrine in the presence of guanethidine (1 μM) and were stimulated six times with trains of 200 pulses at 10 Hz, at intervals of 30 min. CTX CVID at 100 nM was added to the recirculating superfusate between the second and third stimuli, then CTX CVIB at 100 nM was added between the fourth and fifth stimuli. In an additional five experiments, CTX GVIA (100 nM) was applied between the second and third stimulation periods, then CTX CVID (100 nM) was added to the GVIA between the fourth and fifth stimuli.

Protocol 7: effect of the R-type channel blocker SNX-482 or the L-type blocker nifedipine on neurogenic dilations of the uterine artery. Arteries were precontracted with phenylephrine in the presence of guanethidine (1 μM) and were stimulated six times with trains of 200 pulses at 10 Hz, at intervals of 30 min. CTX GVIA (30 nM) was added to the recirculating superfusate between the second and third stimuli, then SNX-482 (100 nM, $n=5$) or nifedipine (10 μM, $n=4$) was added between the fourth and fifth stimuli.

Protocol 8: effect of the T-type blocker $NiCl_2$ on neurogenic dilations of the uterine artery. Arteries were precontracted with phenylephrine in the presence of guanethidine (1 μM) and were stimulated eight times with trains of 200 pulses at 10 Hz, at intervals of 30 min. CTX GVIA (30 nM) was added to the recirculating superfusate between the second and third stimuli, then arteries were treated cumulatively with $NiCl_2$ (100 μM, $n=5$ or 300 μM, $n=4$) and L-NAME (10 μM).

Drugs

ω-Conotoxin GVIA and ω-agatoxin IVA were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) or Alomone Laboratories (Jerusalem, Israel). ω-Conotoxins CVIB and CVID were synthesized by the Centre for Drug Development and Design, University of Queensland (Lewis *et al*., 2000). SNX-482, a peptide from the venom of the tarantula *Hysterocrates gigas* (Newcomb *et al*., 1998), was purchased from Peptide Institute Inc., Osaka, Japan. Toxins were dissolved in Tris-buffered saline containing 0.1% BSA (CTX GVIA, ATX), or in distilled water (CTX CVIB, CTX CVID, SNX-482), and stock solutions were aliquoted and stored at -20°C. Phenylephrine hydrochloride, guanethidine sulphate,

propranolol hydrochloride and N^G-nitro-L-arginine methyl ester were purchased from Sigma-Aldrich; stock solutions were made up in phosphate-buffered saline and were stored at 4°C. Nifedipine was purchased from RBI (Research Biochemicals International, Natick, MA, U.S.A.) and was dissolved in ethanol to produce a stock solution of 0.1 M. Capsaicin was purchased from Sigma-Aldrich, and stock solutions (30 mM) were dissolved in 95% ethanol/5% Tween 80.

Data analysis

Group data are expressed as mean ± s.e.m. Data were analysed by repeated-measures analysis of variance (ANOVA) or analysis of covariance (ANCOVA) with pre-planned single degree of freedom linear contrasts or multiple *post hoc* comparisons with Bonferroni correction. These analyses were done using SPSS for Macintosh version 10.0 (SPSS Inc., Chicago, IL, U.S.A.). Regression analysis was performed using Excel (Excel 2000 for Macintosh, Microsoft). The level of significance was set at $P<0.05$.

Results

Blockade of sympathetic vasoconstrictions by the N-type channel blocker ω-conotoxin GVIA

Isometric contractions of the guinea-pig inferior vena cava produced by field stimulation of sympathetic axons with trains of pulses delivered at 20 Hz were abolished by 10 nM CTX GVIA within 60 min of application. There was a parallel decrease in the magnitude and increase in latency of contractions before complete blockade (Figure 1). At 1 nM, CTX GVIA produced a gradual decrease in magnitude and increase in latency of sympathetic contractions, but did not abolish contractions by 2 h after application. Contractions of the uterine artery produced by stimulation with trains of 200 pulses at 1 or 10 Hz were abolished by 1 nM CTX GVIA within 40–60 min of application (Figure 2a). However, 10 nM CTX GVIA was required to abolish contractions produced by stimulation with trains of 600 pulses at 20 Hz (Figure 2b). CTX GVIA did not change the basal force generated by either the vena cava or the uterine artery.

Partial reduction in neurogenic dilations of uterine artery by the N-type channel blocker ω-conotoxin GVIA

Biphasic relaxations of the uterine artery produced by trains of 200 pulses delivered at 10 Hz were not affected by CTX GVIA at 1 nM (Figure 3a). At 10 nM, CTX GVIA produced a small (15%) but significant decrease in the amplitude of relaxations and reduced the integrated responses by 30% (Figure 3b). The degree of attenuation of neurogenic relaxations was no greater when the dose of CTX GVIA was increased to 30 or 100 nM (Figure 3c, d). CTX GVIA (1–100 nM) did not alter the level of precontraction produced by phenylephrine and guanethidine. Furthermore, the effect of CTX GVIA on neurogenic relaxations was not dependent on the level of precontraction of each artery produced by phenylephrine (regression analysis: amplitude of relaxations, $R=0.18$, $F_{(1,23)}=0.7$, $P=0.4$; area of relaxations, $R=0.07$, $F_{(1,23)}=0.1$, $P=0.7$).

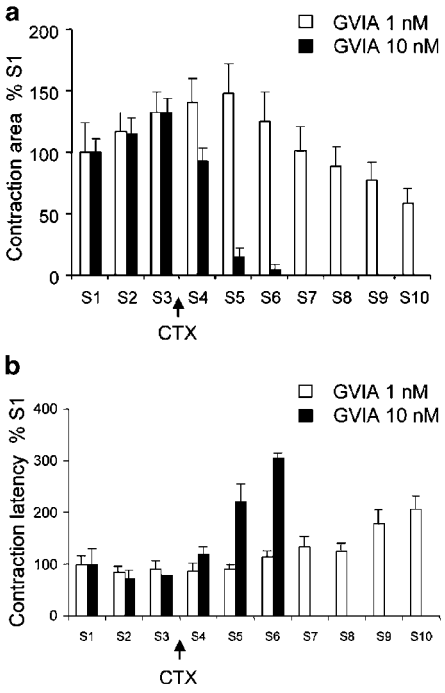

Figure 1 Effects of CTX GVIA on sympathetic contractions of the vena cava. Bars represent mean ± s.e.m. ($n=4$) of the integrated contractile response (a) and the latency of contractions (b) produced by successive periods of stimulation (S1–S10) with 600 pulses at 20 Hz before and after application of CTX GVIA (1 or 10 nM). In each experiment, responses were expressed as % of response produced at S1.

Capsaicin pretreatment did not affect ω-conotoxin GVIA-sensitive or GVIA-resistant neurogenic dilations

To confirm that neurogenic relaxations attenuated by CTX GVIA were due to stimulation of perivascular autonomic neurons and not branches of peptide-containing sensory neurons, uterine arteries were treated with capsaicin *in vitro*. Arteries precontracted with phenylephrine were relaxed almost to the baseline level by an initial application of capsaicin (1–3 μM), which reversed after washout. When capsaicin was reapplied 2–3 h later, only a small contractile response was evident.

Pretreatment with 3 μM capsaicin did not affect either the first phase (maximum amplitude) or second phase (area of response) of relaxations produced by trains of 200 pulses delivered at 10 Hz, compared with vehicle-treated controls. Furthermore, the reductions in both phases of relaxation produced by 30 nM CTX GVIA (Figure 4) were similar in capsaicin and vehicle-treated arteries. Finally, the nitric oxide synthase inhibitor L-NAME (10 μM) was added to confirm that both a nitrergic and non-nitrergic relaxation remained after CTX GVIA. L-NAME abolished the initial relaxation, leaving a slow response, neither phase being affected by capsaicin treatment (Figure 4; repeated-measures ANOVA, no effect of capsaicin: amplitude, $F_{(1,6)}=2.4$, $P=0.17$; area, $F_{(1,6)}=0.03$, $P=0.9$; no interaction between capsaicin, GVIA and L-NAME: amplitude, $F_{(2,6)}=0.1$, $P=0.9$; area, $F_{(2,6)}=0.8$, $P=0.5$). Reapplication of capsaicin at the conclusion of experiments failed to relax the artery. Again, only a small contraction was evoked.

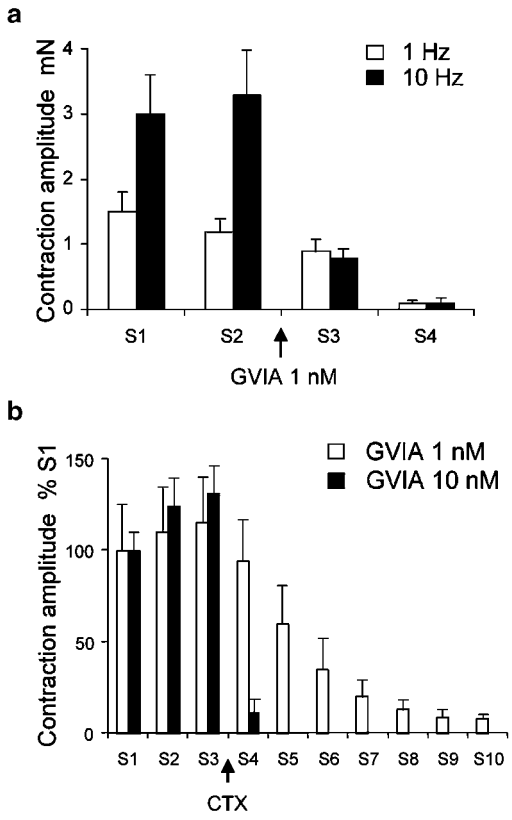

Figure 2 Effects of CTX GVIA on sympathetic contractions of the uterine artery. (a) Contractions produced by trains of 200 pulses delivered at 1 or 10 Hz. Bars represent mean ± s.e.m. ($n = 5$) of the maximum amplitude of contractions before and after addition of 1 nM CTX GVIA. (b) Effects of 1 nM ($n = 4$) and 10 nM ($n = 5$) CTX GVIA on contractions produced by stimulation with 600 pulses at 20 Hz. Bars represent mean ± s.e.m. of contractions expressed as % of amplitude at S1.

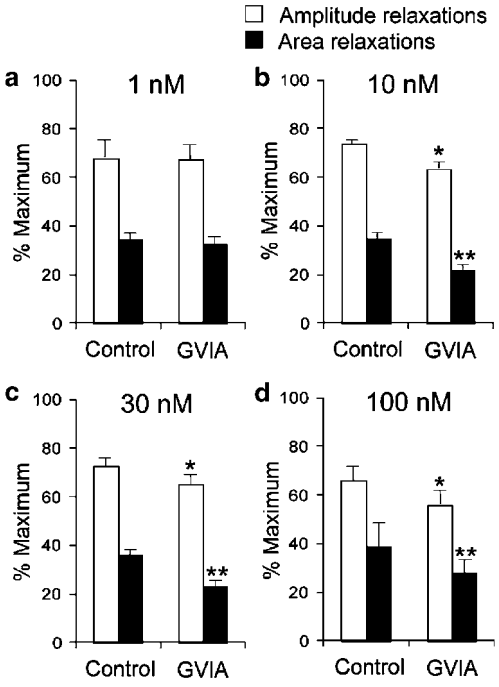

Figure 3 Effect of increasing doses of CTX GVIA on biphasic relaxations of uterine arteries produced by stimulation with trains of 200 pulses at 10 Hz. Bars represent mean ± s.e.m. ($n = 5$) of the maximum amplitude and integrated response produced in the absence (S2, control) and presence (S3, GVIA) of CTX GVIA. Responses in the presence of CTX GVIA were analysed with ANCOVA using the control response as a covariate. Significant effect of GVIA on amplitude ($F_{(3,15)} = 3.9$, $P = 0.03$*) and area ($F_{(3,15)} = 7$, $P = 0.004$**). *Post hoc* comparisons: 1 nM CTX GVIA < 10 nM = 30 nM = 100 nM.

Attenuation of neurogenic dilations by the novel N-type channel blocker ω-conotoxin CVID

CTX CVID at 100 nM partly attenuated both phases of neurogenic relaxations. The degree of attenuation was similar to that produced by 100 nM CTX GVIA in parallel experiments (Figure 5a, b). Furthermore, application of 300 nM CTX CVID after treatment with 100 nM CTX GVIA did not further reduce neurogenic relaxations (Figure 5b). CTX CVID alone did not alter the level of precontraction produced by phenylephrine and guanethidine.

No effect of P/Q-, R- or L-type channel blockers on neurogenic dilations

The P/Q-type blocker ATX IVA at 10 nM did not reduce neurogenic relaxations of the uterine artery (Figure 6a). Furthermore, addition of 100 nM ATX after a supramaximal concentration of CTX GVIA (100 nM) did not produce any additional reduction in either phase of the relaxations (Figure 6b). In separate experiments, the N/P/Q-type blocker CTX CVIB at 100 nM did not further reduce neurogenic relaxations already attenuated by 100 nM CVID (Figure 5a). Neither ATX nor CTX CVIB affected the level of pre-contraction of uterine arteries. Furthermore, SNX-482 (100 nM), a blocker of R-type channels, did not affect neurogenic relaxations remaining after a supramaximal concentration of CTX GVIA (30 nM; Figure 7a), and did not affect the level of pre-contraction. The L-type channel blocker nifedipine (10 μM) produced a small, transient contraction of arteries precontracted with phenylephrine and guanethidine, but did not reduce the magnitude of neurogenic relaxations remaining after CTX GVIA (30 nM; Figure 7b). At the conclusion of experiments, contractions produced by 126 mM KCl were much slower to develop and more transient in nifedipine-treated arteries compared with arteries treated with CTX GVIA alone.

Attenuation of ω-conotoxin-resistant dilations by the T-type calcium channel blocker $NiCl_2$

In the presence of phenylephrine, guanethidine and CTX GVIA (30 nM), $NiCl_2$ (100–300 μM) produced large changes in force generated by segments of uterine artery. There was an initial transient contraction immediately after addition of $NiCl_2$, often followed by a relaxation towards baseline and then a more slowly developing contraction that often exceeded the level prior to $NiCl_2$ treatment. Regardless of the level of contraction after $NiCl_2$ treatment, the CTX GVIA-resistant neurogenic relaxations were reduced significantly in both amplitude and area (Figures 8, 9). Both 100 and 300 μM $NiCl_2$ produced a similar degree of attenuation (repeated-measures ANOVA, no effect of dose of $NiCl_2$: amplitude of relaxations,

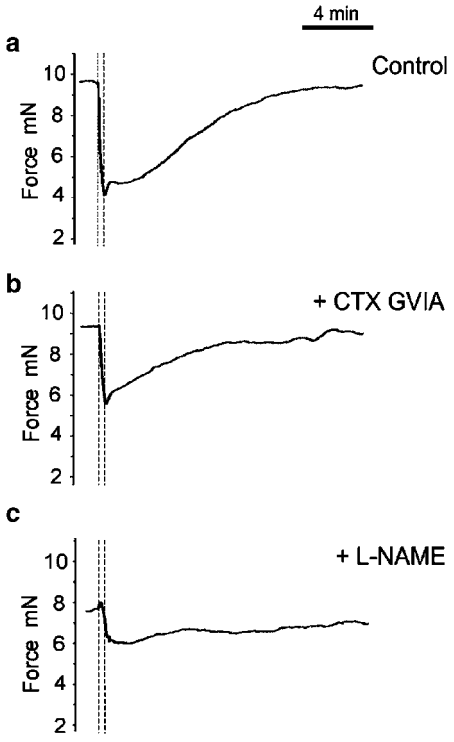

Figure 4 Effect of CTX GVIA on neurogenic relaxations of capsaicin pretreated uterine artery. Representative traces of biphasic relaxations produced by trains of 200 pulses delivered at 10 Hz (between dotted lines) before (a) and after (b) addition of 30 nM CTX GVIA to the superfusate. The relaxation occurring during the stimulation period was greatly reduced after cumulative addition of 10 μM L-NAME (c), leaving a slower relaxation that peaked after cessation of stimulation.

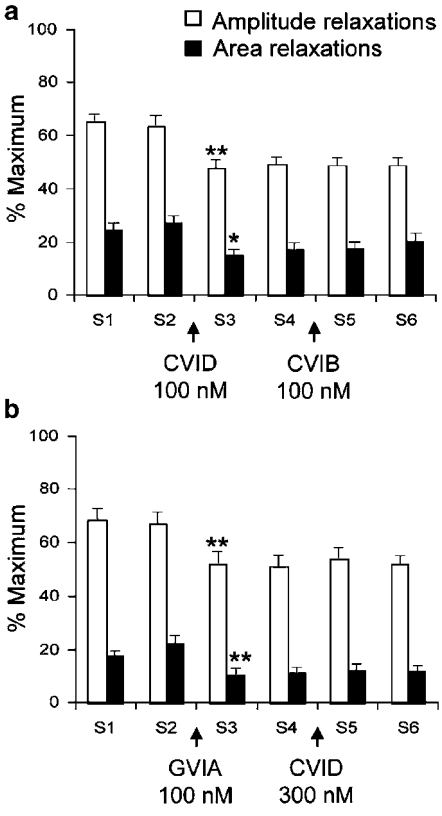

Figure 5 (a) Effect of cumulative addition of CTX CVID 100 nM and CTX CVIB 100 nM on neurogenic relaxations of the uterine artery. Bars represent mean $\pm$ s.e.m. ($n=5$) of the maximum amplitude and area of relaxations. Repeated-measures ANOVA with pre-planned contrasts: significant effect of CTX CVID on amplitude ($F_{(1,4)}=43.5$, $P=0.003$**) and area ($F_{(1,4)}=9.2$, $P=0.04$*); no additional effect of CTX CVIB on amplitude ($F_{(1,4)}=0$, $P=1$) or area ($F_{(1,4)}=2.7$, $P=0.2$). (b) Effect of cumulative addition of CTX GVIA 100 nM and CTX CVID 300 nM on neurogenic relaxations of the uterine artery. Bars represent mean $\pm$ s.e.m. ($n=5$) of the maximum amplitude and area of relaxations. Repeated measures ANOVA with pre-planned contrasts: significant effect of CTX GVIA on amplitude ($F_{(1,4)}=293$, $P=0.0001$**) and area ($F_{(1,4)}=67.2$, $P=0.001$**); no additional effect of CTX CVID on amplitude ($F_{(1,4)}=1$, $P=0.4$) or area ($F_{(1,4)}=2.6$, $P=0.2$).

$F_{(1,7)}=0.004$, $P=0.95$; area of relaxations, $F_{(1,7)}=0.1$, $P=0.75$). Nevertheless, addition of L-NAME demonstrated clearly that both nitrergic and non-nitrergic components of the neurogenic relaxation remained after combined treatment with CTX GVIA and $NiCl_2$ (Figures 8, 9).

Discussion

This study has shown that toxins selective for N-type voltage-gated calcium channels produce different levels of blockade of responses produced by sympathetic vasoconstrictor neurons compared with pelvic vasodilator neurons. Sympathetic noradrenergic constrictions of the uterine artery in response to electrical stimulation at 1 and 10 Hz were abolished by CTX GVIA at 1 nM, the lowest dose used here. Sympathetic constrictions of the inferior vena cava or the uterine artery produced by stimulation with longer trains of pulses at 20 Hz were reduced by 1 nM CTX GVIA, and abolished by 10 nM CTX GVIA. In contrast, neurogenic dilations of the uterine artery produced by 200 pulses at 10 Hz were reduced by less than 50% by doses of CTX GVIA as high as 100 nM. The dilations remaining after CTX GVIA were not further reduced by another N-type blocker, CTX CVID (Lewis *et al*., 2000), nor by the P/Q type channel blockers ATX IVA (Mintz *et al*., 1992; Waterman, 2000) or CTX CVIB (Lewis *et al*., 2000), the L-type blocker nifedipine (Fox *et al*., 1987) or the class E ('R-type') channel blocker SNX-482 (Newcomb *et al*., 1998). The T-type channel blocker Ni^{2+} (Fox *et al*., 1987; Perez-Reyes, 2003) reduced but did not abolish the CTX-resistant dilations.

N-type voltage-gated calcium channels on sympathetic vasoconstrictor neurons

Sympathetic constrictions of both the uterine artery and the vena cava appear to be mediated by calcium entering solely *via* N-type channels, regardless of the stimulation frequency in the range 1–20 Hz, and regardless of the transmitters involved. We previously hypothesized that constriction of the uterine artery at 10 Hz was due to release of NA from two populations of synaptic vesicles, exocytosis from one population being sensitive to blockade by BoNTA and the second resistant to BoNTA (Morris *et al*., 2002). Furthermore, we proposed that sympathetic constrictions of the vena cava produced by stimulation at 20 Hz were mediated by release of NA from a

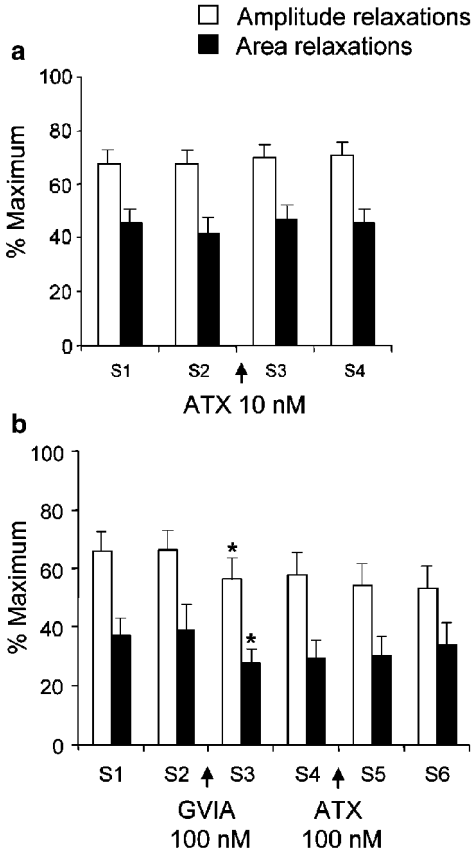

Figure 6 No effect of ATX on neurogenic relaxations of the uterine artery. (a) Bars represent mean ± s.e.m. ($n = 5$) of the maximum amplitude and area of relaxations before and after treatment with 10 nM ATX. Repeated-measures ANOVA: no effect of ATX 10 nM on amplitude ($F_{(1,4)} = 5.9$, $P = 0.07$) or area ($F_{(1,4)} = 1$, $P = 0.4$). (b) Bars represent mean ± s.e.m. ($n = 5$) of the maximum amplitude and area of relaxations before and after cumulative addition of 100 nM CTX GVIA and 100 nM ATX. Repeated-measures ANOVA with pre-planned contrasts: significant effect of CTX GVIA on amplitude ($F_{(1,4)} = 16.5$, $P = 0.02$*) and area ($F_{(1,4)} = 17.1$, $P = 0.01$*); no further effect of ATX 100 nM on amplitude ($F_{(1,4)} = 2.7$, $P = 0.2$) or area ($F_{(1,4)} = 2.7$, $P = 0.2$).

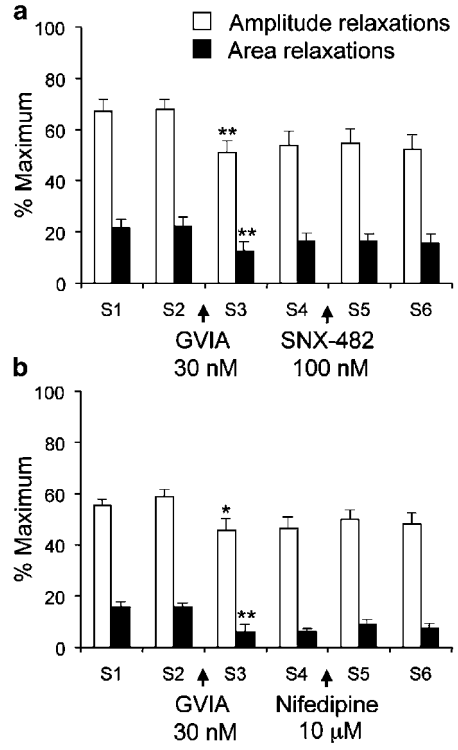

Figure 7 No effect of SNX-482 100 nM (a) or nifedipine 10 μM (b) on neurogenic relaxations resistant to CTX GVIA (30 nM). Bars represent mean ± s.e.m. ($n = 5$ for SNX-482, $n = 4$ for nifedipine) of the amplitude and area of relaxations expressed as % maximum. Repeated-measures ANOVA with pre-planned contrasts: (a) significant effect of CTX GVIA on amplitude ($F_{(1,4)} = 244$, $P = 0.0001$**) and area ($F_{(1,4)} = 38$, $P = 0.003$**); no effect of SNX-482 on amplitude ($F_{(1,4)} = 2.3$, $P = 0.2$) or area ($F_{(1,4)} = 2.4$, $P = 0.2$). (b) Significant effect of CTX GVIA on amplitude ($F_{(1,3)} = 31$, $P = 0.01$*) and area ($F_{(1,3)} = 64$, $P = 0.004$**); significant increase in amplitude after nifedipine ($F_{(1,3)} = 73$, $P = 0.003$); no effect of nifedipine on area ($F_{(1,3)} = 1.8$, $P = 0.3$).

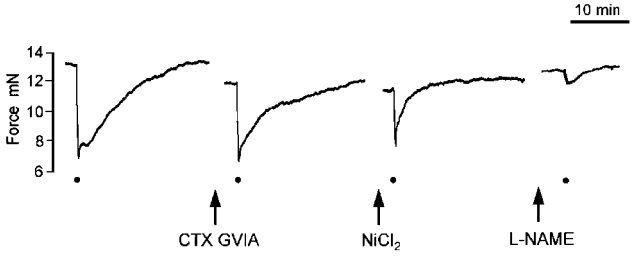

Figure 8 Effect of $NiCl_2$ on CTX GVIA-resistant neurogenic relaxations of the uterine artery. Representative trace showing relaxations produced by electrical stimulation with trains of 200 pulses at 20 Hz (at black dots) before and 20 min after addition of 30 nM CTX GVIA, 40 min after cumulative addition of 100 μM $NiCl_2$ and 40 min after addition of 10 μM L-NAME.

BoNTA-sensitive pool, and release of both NA and NPY from a BoNTA-resistant pool (Morris *et al*., 2002). It is likely that the two transmitter pools correspond to small and large synaptic vesicles, respectively. The current study aimed to test if different subtypes of calcium channels were involved in transmitter release from the two pools. This does not appear to be the case.

There are other vascular beds where N-type calcium channels alone seem to be involved in calcium entry into sympathetic neurons (Ren *et al*., 1994; Matthew & Wadsworth, 1997; Morris *et al*., 1998; Yang & Chiba, 2000). However, there are also reports of a component of sympathetic vasoconstrictions mediated by release of ATP and noradrenaline that is resistant to CTX GVIA (Brock & Cunnane, 1999; Tanaka *et al*., 1999; Nedergaard, 2000; Waterman, 2000). In the rabbit aorta, the CTX GVIA-resistant release of NA was more prevalent at higher stimulation frequencies (Nedergaard, 2000). Similarly, both purinergic and noradrenergic transmission from sympathetic neurons to the guinea-pig vas deferens showed a CTX GVIA-resistant component at higher frequencies of stimulation that was not evident at low stimulation frequencies (Smith & Cunnane, 1996; 1998). It is probable that this 'residual release' (Smith & Cunnane, 1996) is due to exocytosis from a pool of synaptic vesicles distinct from those releasing transmitter at low stimulation frequencies. Thus, differential involvement of calcium channel subtypes in release of different pools of neurotransmitters may exist in some other sympathetic neurons.

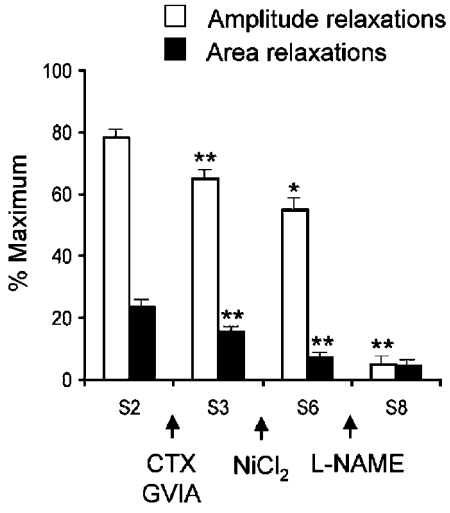

Figure 9 Attenuation by $NiCl_2$ of CTX GVIA-resistant neurogenic relaxations of the uterine artery. Bars represent mean ± s.e.m. of amplitude and area of relaxations in response to stimulation with 200 pulses at 10 Hz. Group data pooled from nine experiments using 100 μM ($n=5$) or 300 μM ($n=4$) $NiCl_2$. Responses before (S2) and after maximal reduction produced by cumulative addition of 30 nM CTX GVIA between S2 and S3, $NiCl_2$ added between S4 and S5, and 10 μM L-NAME added between S6 and S7. Repeated-measures ANOVA with pre-planned contrasts: significant effect of CTX GVIA on amplitude ($F_{(1,7)}=56$, $P=0.0001$**) and area ($F_{(1,7)}=80$, $P=0.0001$**); significant effect of $NiCl_2$ on amplitude ($F_{(1,7)}=210$, $P=0.02$*) and area ($F_{(1,7)}=16$, $P=0.005$**); significant effect of L-NAME on amplitude ($F_{(1,7)}=133$ $P=0.0001$**); no effect of L-NAME on area ($F_{(1,7)}=5$, $P=0.07$).

N-type voltage-gated calcium channels on pelvic vasodilator neurons

Vasodilation of the uterine artery in response to trains of pulses is mediated by neuronal NO and one or more neuropeptides (Morris, 1993; Morris *et al*., 2001). It is clear that N-type calcium channels partly mediate both components of the biphasic response. However, doses of CTX GVIA up to 100 nM reduced responses produced by NO and peptides by less than 50%. Indeed, the maximum reduction by CTX GVIA was achieved at a dose of 10 nM. Thus, involvement of N-type channels in increasing intracellular calcium levels was not selective for either synthesis of NO by nNOS or exocytosis of neuropeptides from large vesicles. N-type channels have been reported to mediate calcium entry for NO synthesis by vasodilator neurons in the dog corpus cavernosum (Okamura *et al*., 2001), but not the sheep middle cerebral artery (Matthew & Wadsworth, 1997).

Previously, we concluded that the slow dilation produced by electrical stimulation of uterine artery segments *in vitro* was mediated by the peptides VIP and/or CGRP released from pelvic autonomic neurons whose axons form a dense plexus at the adventitia-medial junction (Morris, 1993; Anderson *et al*., 1997). As receptor antagonists for the dilator actions of VIP or CGRP on the uterine artery are not available (Morris & Murphy, 1989; Anderson *et al*., 1997), evidence for the role of neuropeptides in neurogenic vasodilation rests with the selective blockade of slow, non-nitrergic relaxations by the endopeptidase trypsin (Morris, 1993). Here we tested the effect of capsaicin on the slow vasodilator response before and after CTX GVIA treatment to determine directly if peptide release from sensory neurons contributed at all to the neurogenic dilations (Holzer, 1992), and whether partial reduction of the peptide-mediated component by CTX GVIA was a selective effect on autonomic or sensory neurons. Capsaicin pretreatment did not reduce neurogenic dilations to electrical stimulation and did not alter the inhibition produced by CTX GVIA. Thus, the N-type channels involved in release of vasodilator neurotransmitters are likely to be entirely on autonomic vasodilator neurons.

CTX GVIA-resistant channels mediating autonomic vasodilation

The vasodilator responses resistant to CTX GVIA are unlikely to be due to calcium entry *via* P/Q- or L-type voltage-gated channels, as high concentrations of ATX IVA (Mintz *et al*., 1992), CTX CVIB (Lewis *et al*., 2000) or nifedipine (Fox *et al*., 1987) did not further reduce dilations remaining after a supra-maximal dose of CTX GVIA. Given the recent identification of variant N-type channels in cranial parasympathetic preganglionic neurons using the toxin CTX CVID (Adams *et al*., 2003), we tested the effects of CTX CVID on pelvic vasodilator neurons. However, CTX CVID did not appear to produce any blockade in addition to that attributable to blockade of standard N-type channels (Lewis *et al*., 2000; Wright *et al*., 2000; Scott *et al*., 2002; Smith *et al*., 2002). Thus, the role of variant N-type channels in mediating CTX GVIA-resistant responses to stimulation of some parasympathetic neurons (Adams *et al*., 2003) does not seem to be universal. It is possible that the CTX GVIA-resistant channel in our study is similar to the ω-grammatoxin-sensitive channel reported in preganglionic pelvic neurons in male guinea-pigs (Smith & Cunnane, 1999). However, another 'R-type' blocker, SNX-482 (Newcomb *et al*., 1998), failed to reduce the CTX GVIA-resistant vasodilators of the uterine artery.

The only calcium channel blocker used here that reduced the CTX GVIA-resistant neurogenic dilations was $NiCl_2$, a blocker of low voltage-activated T-type calcium channels (Fox *et al*., 1987; Perez-Reyes, 2003). As the attenuation by $NiCl_2$ occurred in the presence of a supramaximal dose of CTX GVIA, this effect is unlikely to be due to additional blockade of N-type channels. The effect of $NiCl_2$ on vascular tone in our preparations is consistent with a previous demonstration of T-type calcium channels on vascular smooth muscle cells (Xi *et al*., 2002), and it is possible that the attenuation of neurogenic dilations of the uterine artery by $NiCl_2$ is a postsynaptic action. However, the attenuation was apparent regardless of the level of precontraction after nickel treatment. Thus, it is likely that T-type calcium channels are present on the terminals of pelvic vasodilator neurons and contribute to calcium entry leading to release of both NO and neuropeptides. This conclusion is consistent with reports of T-type channels on the soma of pelvic neurons in male rats (Zhu *et al*., 1995; Lee *et al*., 2002), although those channels were located on noradrenergic but not nitrergic pelvic neurons (Zhu *et al*., 1995).

In conclusion, this study has not supported our hypothesis that different subtypes of voltage-gated calcium channels are involved in release of co-transmitters from the same autonomic vasomotor neuron. However, the results clearly demonstrate

that sympathetic vasoconstrictor and pelvic vasodilator neurons use different subtypes of channels, even when innervating the same artery (see Matthew & Wadsworth, 1997; see Ino *et al*., 2001). Finally, we have demonstrated a component of transmitter release from pelvic vasodilator neurons that is resistant to N-, P/Q-, L-, R- and T-type calcium channel blockers. The resistant channel appears to be different from the variant N-type channel reported in cranial parasympathetic neurons of rats. This diversity of calcium channels between different populations of vasomotor neurons provides further evidence for the heterogeneity of mechanisms of neurotransmission that match the pattern of activity of each population of neurons to its physiological role in autonomic control of the cardiovascular system (Morris *et al*., 1998; 2001; 2002).

This work was supported by the National Health and Medical Research Council of Australia (Project Grant 000023; Principal Research Fellowship 160083 to J. Morris) and a National Heart Foundation of Australia Vacation Scholarship to D. Ozols (VS 02A 0921). We acknowledge the excellent technical assistance of Pat Vilimas.

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(Received November 11, 2003
Revised January 7, 2004
Accepted January 23, 2004)