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Significant role of neuronal non-N-type calcium channels in the sympathetic neurogenic contraction of rat mesenteric artery

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1 The possible involvement of pre-junctional non-N-type Ca^{2+} channels in noradrenaline (NA)-mediated neurogenic contraction by electrical field stimulation (EFS) was examined pharmacomechanically in the isolated rat mesenteric artery.

2 EFS-generated contraction of endothelium-denuded mesenteric artery was frequency-dependent (2–32 Hz) and was abolished by tetrodotoxin (TTX, 1 μM), guanethidine (5 μM) or prazosin (100 nM), indicating that NA released from sympathetic nerve endings mediates the contractile response.

3 NA-mediated neurogenic contractions to lower frequency stimulations (2–8 Hz) were almost abolished by an N-type Ca^{2+} channel blocker, ω-conotoxin-GVIA (1 μM) whereas the responses to higher frequency stimulations (12–32 Hz) were less sensitive to ω-conotoxin-GVIA. The ω-conotoxin-GVIA-resistant component of the contractile response to 32 Hz stimulation was inhibited partly (10–20%) by ω-agatoxin-IVA (10–100 nM; concentrations which are relatively selective for P-type channels) and to a greater extent by ω-agatoxin-IVA (1 μM) and ω-conotoxin-MVIIC (3 μM), both of which block Q-type channels at the concentrations used.

4 ω-Agatoxin-IVA (10–100 nM) alone inhibited 32 Hz EFS-induced contraction by 10∼20% whereas ω-conotoxin-MVIIC (3 μM) alone inhibited the response by ∼60%.

5 These ω-toxin treatments did not affect the contractions evoked by exogenously applied NA.

6 These findings show that P- and Q-type as well as N-type Ca^{2+} channels are involved in the sympathetic neurogenic vascular contraction, and suggest the significant role of non-N-type Ca^{2+} channels in NA release from adrenergic nerve endings when higher frequency stimulations are applied to the nerve.

Keywords: Ca^{2+} channels, N-type; Ca^{2+} channels, P-type; Ca^{2+} channels, Q-type; ω-conotoxin-GVIA; ω-agatoxin-IVA; ω-conotoxin-MVIIC; sympathetic nerve transmission; rat mesenteric artery

Abbreviations: EFS, electrical field stimulation; NA, noradrenaline; TTX, tetrodotoxin

Introduction

Noradrenaline (NA) released from adrenergic nerve endings plays the predominant role in the neurogenic vascular contraction. The release of neurotransmitters including the catecholamine requires the influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels at peripheral nerve endings (Smith & Augustine, 1988).

As to the Ca^{2+} channels responsible for the peripheral sympathetic nerve transmission in vascular tissues, a significant role for N-type Ca^{2+} channels has been indicated based on the high susceptibility to inhibition by ω-conotoxin-GVIA (Olivera *et al*., 1984), a selective N-type Ca^{2+} channel blocker (Hirning *et al*., 1988; McCleskey *et al*., 1987), in rabbit ear artery (Zygmunt & Högestätt, 1993) and pulmonary artery (Lundy & Frew, 1994); rat mesenteric artery (Pruneau & Angus, 1990; Wright & Angus, 1996) and tail artery (Clasbrummel *et al*., 1989); and dog splenic artery (Ren *et al*., 1994). In contrast, in non-vascular tissues, substantial contribution of non-N-type Ca^{2+} channels (P- and Q-type Ca^{2+} channels) is proposed in the control of sympathetic neurotransmission in mouse vas deferens (Wright & Angus, 1996) and parasympathetic neurotransmission in rat urinary bladder (Frew & Lundy, 1995). However, in the vasculature, there is little information available on the role of non-N-type Ca^{2+} channels in the release of NA, although sympathetic neuromuscular transmission is crucially relevant in the maintenance of vascular resistance (Pruneau & Angus, 1990; Ren *et al*., 1994).

The aim of the present study was to further investigate pharmacomechanically the possible presence of non-N-type Ca^{2+} channels at the adrenergic neuroeffector junctions in rat mesenteric artery. For this purpose, the effects of ω-conotoxin-GVIA (N-type channel blocker) ω-agatoxin-IVA (P-type channel blocker) (Mintz *et al*., 1992a,b) and ω-conotoxin-MVIIC (N-, P- and Q-type channel blocker) (Hillyard *et al*., 1992; Wheeler *et al*., 1994) were examined on the contractile responses to electrical field stimulation (EFS) and exogenous NA in the isolated rat mesenteric artery.

Methods

Male Wistar rats (Wistar/ST: supplied from Nihon SLC Co. Ltd., Hamamatsu-City, Shizuoka, Japan) were housed under controlled conditions (temperature 21–22°C, relative air humidity 50±5%). Food and water were available *ad libitum* to all animals. This study was conducted in accordance with the Guideline for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory

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Animals of Toho University School of Pharmaceutical Sciences (which is accredited by the Ministry of Education, Science, Sports and Culture, Japan).

Preparation

Superior mesenteric arteries with an average outer diameter of 500–700 μm were isolated from male Wistar rats (200–300 g) and placed in normal Tyrode's solution of the following composition: (mM) NaCl, 158.3; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 10.0 and glucose, 5.6 (pH = 7.4). The arteries were cleared of connective tissue and cut into ring segments of about 1 mm in length. Endothelium was removed by rubbing gently the intimal surface with moistened cotton strings. Removal of endothelium was verified by abolishment of the relaxation to acetylcholine (ACh) (10 μM). Mesenteric artery rings were mounted using stainless steel hooks in a 4-ml organ bath (Micro Tissue Organ Bath: MTOB-1, Labo Support, Suita-City, Japan) containing normal Tyrode's solution, which was aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 36.5 ± 0.5°C. Tension changes were measured isometrically as reported previously (Tanaka *et al*., 1998). The resting tension was adjusted to 0.75 g. The rings were allowed to equilibrate in the bathing media for 40–60 min, during which time the media were replaced every 15–20 min.

Electrical field stimulation (EFS)

Most of the arterial rings were placed between stimulating electordes made of platinum. A train of 0.2 ms square pulses of supramaximal intensity (50 V) was applied transmurally at a frequency between 2–32 Hz for 30 s. The stimulus pulses were delivered by an electronic stimulator (SEN-3301, Nihon Kohden Kogyo, Tokyo, Japan). The interval between electrical stimulations was at least 5 min. When 2–3 reproducible contractions were obtained, ω-toxin Ca^{2+} channel blockers were added to the bath solution and present in the solution for 10 min until next electrical stimulation was applied. The neurogenic characteristics of the constrictor responses to EFS were confirmed at the end of every experiment by the abolition of the responses in the presence of tetrodotoxin (TTX) (1 μM) (Frew & Lundy, 1995; Zygmunt & Högestätt, 1993). All the experiments were carried out in the presence of indomethacin (3 μM) to prevent possible effects of endogenous prostaglandins.

Concentration-response relationships for NA-induced contraction

Concentration-response relationships for exogenous NA-induced contraction were obtained in the absence and presence of ω-toxin Ca^{2+} channel blockers. The ω-toxins were added to the bath solution 10 min before the second cumulative application of NA.

Drugs

ω-Conotoxin-GVIA, ω-agatoxin-IVA, ω-conotoxin-MVIIC (Peptide Research Foundation, Minoh, Osaka, Japan); tetrodotoxin (TTX) (Sankyo, Tokyo). All other chemicals used in the present study were commercially available and of reagent grade. All drugs are expressed in molar concentrations in the bathing solution.

Data collection and analysis

Data were collected and analysed using a MacLab/400™ and Chart™ (Version 3.5) software (ADInstruments Japan, Tokyo, Japan).

To construct concentration-response relationships for exogenous NA-induced contractions, the percentage of NA-induced contraction was calculated considering the basal tension level before application of NA as 0% and the maximum tension level obtained by 30 μM NA as 100%. Data were plotted as a function of drug concentration and fitted to the equation:

$$E = E_{max} \times A^{n_H} / (EC_{50}^{n_H} + A^{n_H})$$

where E is % contraction at a given NA concentration, E_{max} is the maximum contraction, A is the concentration of NA, n_H is the slope function and EC_{50} is the effective NA concentration that produces a 50% response (Parker & Waud, 1971). The curve fitting was carried out using GraphPad Prism™ (version 2.01) (GraphPad Software, Inc., San Diego, CA, U.S.A.). The EC_{50} values were obtained from individual concentration-response curves and converted to logarithmic values (pD_2) for statistical analysis.

All data are presented as mean values ± s.e.mean and n refers to the number of experiments. The significance of the difference between mean values was evaluated by paired t-test or one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test with GraphPad Prism™. A P value less than 0.05 was considered statistically significant.

Results

EFS-evoked contraction and effects of ω-conotoxin-GVIA

Figure 1a shows an experimental trace illustrating the contraction of an endothelium-denuded rat mesenteric artery preparation in response to electrical field stimulation (EFS). EFS applied to the mesenteric artery generated contractions in a frequency-dependent manner (2–32 Hz). The contractions were abolished by TTX (1 μM), guanethidine (5 μM) or prazosin (100 nM), indicating that EFS-evoked contraction of mesenteric artery under this condition is neurogenic and mediated *via* release of NA from sympathetic nerve endings leading to the activation of α_1-adrenoceptors in vascular smooth muscle cells.

As shown in Figure 1a, ω-conotoxin-GVIA (1 μM), which blocks N-type Ca^{2+} channels, diminished EFS-induced contractions. The inhibition by ω-conotoxin-GVIA (1 μM) was almost complete for the contractile responses to lower frequency stimulations (2–8 Hz), which indicates the predominant role of N-type Ca^{2+} channels in the neurogenic responses. In contrast, the contractile responses to higher frequency stimulations (12–32 Hz) were more resistant to the treatment with ω-conotoxin-GVIA (1 μM) than the responses to lower frequency EFS (2–8 Hz). Figure 1b shows the summarized results of the inhibitory effects of ω-conotoxin-GVIA (1 μM) on the contractile responses to EFS with varied frequency. Pronounced suppression by ω-conotoxin-GVIA (1 μM) was again evident in the lower frequency range (2–8 Hz). If we assume that N-type Ca^{2+} channels are completely blocked by ω-conotoxin-GVIA at this concentration (1 μM), this finding indicates that activation of non-N-type Ca^{2+} channels contributes substantially to the neurogenic contractions generated by higher frequency of EFS.

Effects of ω-agatoxin-IVA and ω-conotoxin-MVIIC on non-N-type Ca^{2+} channel-mediated contraction

To determine pharmacologically whether activation of P- and Q-type Ca^{2+} channels is involved in non-N-type Ca^{2+} channel-mediated vascular contraction, the effects of ω-agatoxin-IVA (P-type channel blocker) and ω-conotoxin-MVIIC (N-, P- and Q-type channel blocker) were examined on the ω-conotoxin-GVIA-resistant component of the contraction evoked by 32 Hz EFS. The extent of the inhibition by ω-conotoxin-GVIA (1 μM) varied depending on the tissue used (5–70%) and the average inhibition was 27.6±3.1% ($n=29$). Thus, about 70% (72.4%) of the response was assumed to be resistant to the blockade of the pre-junctional N-type Ca^{2+} channels. Further addition of ω-agatoxin-IVA (100 nM) partially suppressed the ω-conotoxin-GVIA-resistant contractile component: ω-Agatoxin-IVA (100 nM) reduced the ω-conotoxin-GVIA (1 μM)-resistant contractile component from 60% (62.8±5.1%, $n=10$; control, 100%) to 45% (44.6±5.4%, $n=10$). If we assume that N-type channels are completely blocked by ω-conotoxin-GVIA (1 μM) and ω-agatoxin-IVA blocks only P-type channels at this concentration (100 nM), contribution of P-type Ca^{2+} channels to the total contractile response to 32 Hz EFS was estimated to be ≈20%.

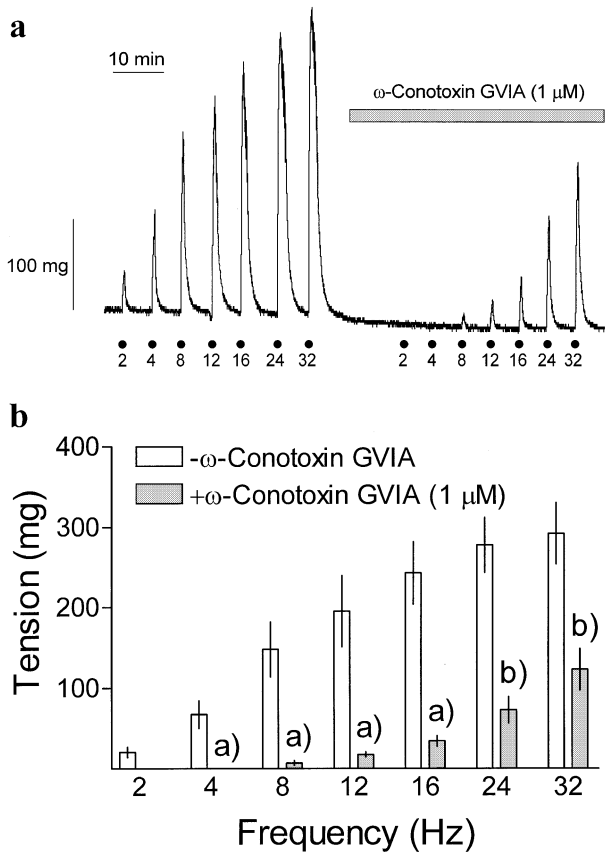

Figure 1 Inhibitory effects of ω-conotoxin-GVIA on electrical field stimulation (EFS)-evoked contraction of rat mesenteric artery. (a) A representative mechanical trace showing the inhibitory effects of ω-conotoxin-GVIA (1 μM). EFS (train, 2–32 Hz; duration, 0.2 ms; periods, 30 s; voltage, 50 V) was applied to rat mesenteric artery denuded of endothelium to generate contraction. (b) Summarized results of the inhibitory effects of ω-conotoxin-GVIA (1 μM) on the contractile responses to EFS with varied frequency. Data are mean values±s.e.mean of four experiments. Significant difference from the control values: [a)] $P<0.05$, [b)] $P<0.01$.

Figure 2 is a trace showing the inhibitory effects of sequentially applied ω-toxins (ω-conotoxin-GVIA, 1 μM; ω-agatoxin-IVA, 100 nM; and ω-conotoxin-MVIIC, 3 μM) on the contraction evoked by 32 Hz EFS. ω-Conotoxin-GVIA (1 μM) alone reduced the contraction to 61.0±3.8% ($n=4$) (control, 100%), showing that about 40% of the response is due to the activation of N-type channels in this series of experiments. Further application of ω-agatoxin-IVA (100 nM) significantly attenuated the residual response to 42.7±7.2% ($n=4$) ($P<0.05$) (Figures 2 and 3a). On the other hand, in the absence of ω-conotoxin-GVIA, ω-agatoxin-IVA (100 nM) alone attenuated the contraction evoked by 32 Hz stimulation to 78.6±7.7% ($n=8$) (control=100%), which indicates again that contribution of P-type channels is about 20% under this condition (Figure 3b).

Low concentrations (<100 nM) of ω-agatoxin-IVA has been used as a relatively selective antagonist of P-type Ca^{2+} channels in pharmacomechanical studies (Frew & Lundy, 1995; Wright & Angus, 1996). However, ω-agatoxin-IVA at concentrations higher than 200 nM may block Q-type channels (Wheeler *et al*., 1994). Thus, inhibitory actions by 100 nM ω-agatoxin-IVA might be mediated partly *via* blockade of neuronal Q-type channels. To further confirm that P-type channels substantially contribute to sympathetic neurogenic contraction, effects of low concentration (10 nM) of ω-agatoxin-IVA were examined on the vascular contraction elicited by 32 Hz EFS. Following blockade of N-type Ca^{2+} channels with ω-conotoxin-GVIA (1 μM), 10 nM ω-agatoxin-IVA inhibited further the residual response from 78.8±3.2 to 67.2±3.6% ($n=9$, $P<0.05$) (Figure 3c). Thus, contribution of P-type Ca^{2+} channels to the contractile response is significant, and the degree of the contribution was estimated to be about 10% (11.6%). Furthermore, in the absence of ω-conotoxin-GVIA, 10 nM ω-agatoxin-IVA alone significantly inhibited the response by 10% (10.3%) ($n=4$) (Figure 3d), indicating again the substantial role of P-type Ca^{2+} channels in the response. Both in the absence and presence of ω-conotoxin-GVIA (1 μM), the degree of inhibition produced by 100 nM ω-agatoxin-IVA and that by 10 nM of the toxin did not differ significantly from each other ($P>0.05$ for both). This implies that inhibitions by both concentrations of ω-agatoxin-IVA are practically the same and due to the blockade of P-type channel only. The ω-agatoxin-IVA (10, 100 nM)-insensitive contractile

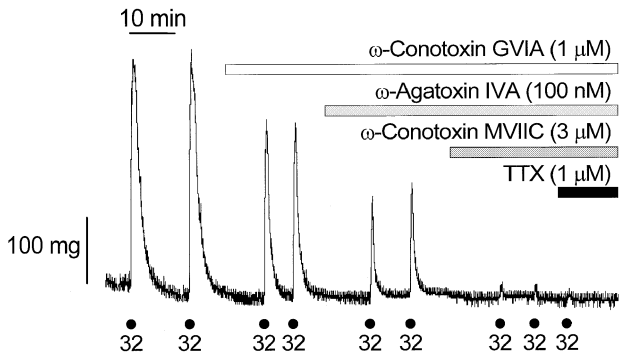

Figure 2 A representative mechanical trace showing the inhibitory effects of sequentially applied ω-toxins on the contractions of rat mesenteric artery elicited by 32 Hz EFS. EFS (train, 32 Hz; duration, 0.2 ms; periods, 30 s; voltage, 50 V) was applied to rat mesenteric artery denuded of endothelium to generate contraction. ω-Conotoxin-GVIA (1 μM), ω-agatoxin-IVA (100 nM) and ω-conotoxin-MVIIC (3 μM) were applied sequentially to the bath solution. TTX; tetrodotoxin, 1 μM.

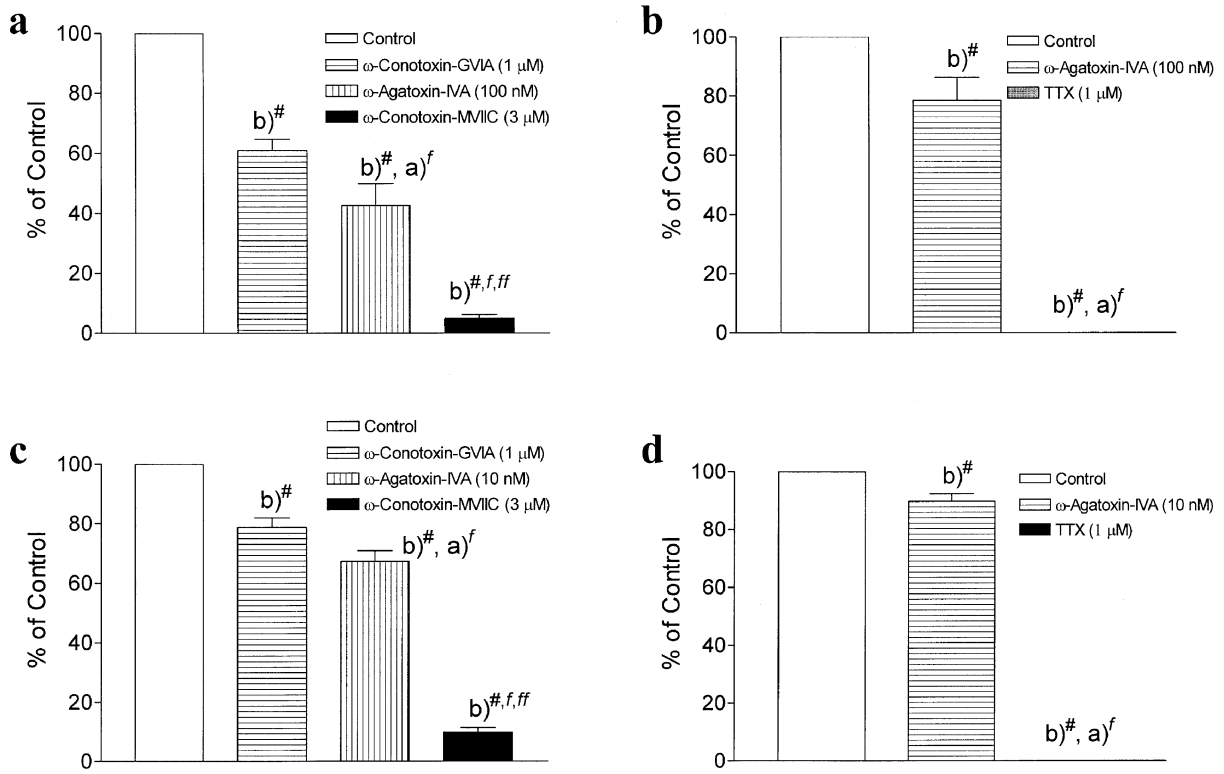

Figure 3 Inhibitory effects of ω-toxins on the contractile responses to 32 Hz EFS. (a) Summarized inhibitory effects sequentially applied ω-toxins on the contractions of rat mesenteric artery elicited by 32 Hz EFS shown in Figure 2. ω-Conotoxin-GVIA (1 μM), ω-agatoxin-IVA (100 nM) and ω-conotoxin-MVIIC (3 μM) were applied sequentially to the bath solution. Data are mean values ± s.e.mean of four experiments. Significant difference from the corresponding values: b)$^{\#}P<0.01$ vs control response in the absence of ω-toxins; a)$^{f}P<0.05$, b)$^{f}P<0.01$ vs the response in the presence of ω-conotoxin-GVIA (1 μM) b)$^{ff}P<$ 0.01 vs the response in the presence of ω-conotoxin-GVIA (1 μM) plus ω-agatoxin-IVA (100 nM). (b) Inhibitory effects of ω-agatoxin-IVA on the contractions of rat mesenteric artery elicited by 32 Hz EFS. ω-Agatoxin-IVA (100 nM) alone was applied to the bath solution. TTX (tetrodotoxin, 1 μM) abolished ω-agatoxin-IVA-resistant contractile component. Data are mean values ± s.e.mean of eight experiments. Significant difference from the corresponding values: b)$^{\#}P<0.01$ vs control response in the absence of ω-agatoxin-IVA; a)$^{f}P<0.05$, vs the response in the presence of agatoxin-IVA (100 nM). (c) Summarized inhibitory effects sequentially applied ω-toxins on the contractions of rat mesenteric artery elicited by 32 Hz EFS. ω-Conotoxin-GVIA (1 μM), ω-agatoxin-IVA (10 nM) and ω-conotoxin-MVIIC (3 μM) were applied sequentially to the bath solution. Data are mean values ± s.e.mean of nine experiments. Significant difference from the corresponding values: b)$^{\#}P<0.01$ vs control response in the absence of ω-toxins; a)$^{f}P<0.05$, b)$^{f}P<0.01$ vs the response in the presence of ω-conotoxin-GVIA (1 μM); b)$^{ff}P<0.01$ vs the response in the presence of ω-conotoxin-GVIA (1 μM) plus ω-agatoxin-IVA (10 nM). (d) Inhibitory effects of ω-agatoxin-IVA on the contractions of rat mesenteric artery elicited by 32 Hz EFS. ω-Agatoxin-IVA (10 nM) alone was applied to the bath solution. TTX (tetrodotoxin, 1 μM) abolished ω-agatoxin-IVA (10 nM)-resistant contractile component. Data are mean values ± s.e.mean of four experiments. Significant difference from the corresponding values. b)$^{\#}P<0.01$ vs control response in the absence of ω-agatoxin-IVA; a)$^{f}P<0.05$, vs the response in the presence of agatoxin-IVA (10 nM).

component was abolished by the treatment with TTX (1 μM) (Figure 3b,d).

In the combined presence of ω-conotoxin-GVIA (1 μM) and ω-agatoxin-IVA (10 or 100 nM), the residual component was significantly reduced by further addition of ω-conotoxin-MVIIC (3 μM) to 5.0 ± 1.3% ($n=4$) ($P<0.01$) (Figures 2 and 3a) or to 9.8 ± 1.6% ($n=9$) ($P<0.01$) (Figure 3c). Although ω-conotoxin-MVIIC blocks non-selectively N-, P- and Q-type Ca^{2+} channels, in the presence of ω-conotoxin-GVIA (1 μM) plus ω-agatoxin-IVA (10 or 100 nM), the contractile component sensitive to ω-conotoxin-MVIIC (3 μM) is ascribed to the activation of neuronal Q-type Ca^{2+} channels. Thus, about 40–60% of the total contraction is assumed to be attributable to the activation of Q-type Ca^{2+} channels under these conditions (32 Hz EFS) (Figure 3a,c).

ω-Conotoxin-GVIA (1 μM)-insensitive component of the contraction evoked by the stimulation of 32 Hz EFS was significantly attenuated from 63.5 ± 12.4 to 9.0 ± 3.1% ($n=4$ for each, $P<0.05$, total response = 100%) by the treatment with ω-conotoxin-MVIIC (3 μM) alone (Figure 4a). High concentration of ω-agatoxin IVA (1 μM) also significantly diminished the residual response remaining in the presence of ω-conotoxin-GVIA (1 μM) to the similar degree as ω-conotoxin-MVIIC (3 μM) (from 84.5 ± 4.4 to 14.1 ± 3.9%, $n=5$ for each, $P<0.05$) (Figure 4b). These findings indicate again that activation of P- and Q-type Ca^{2+} channels is involved in non-N-type Ca^{2+} channel-mediated neurogenic vascular contraction.

ω-Conotoxin-MVIIC (3 μM) alone attenuated the contraction evoked by 32 Hz EFS to 44.0 ± 9.2% ($n=4$, $P<0.01$) (control = 100%). The remaining contraction was reduced to 11.2 ± 2.6% ($n=4$, $P<0.01$) by further addition of ω-conotoxin-GVIA (1 μM) (Figure 5). This finding implies that suppression of N-type Ca^{2+} channels is small or not involved in the inhibitory action of ω-conotoxin-MVIIC (3 μM) on NA-mediated neurogenic contraction of rat mesenteric artery.

Effects of ω-toxins on the contraction evoked by exogenous NA

The effects of the ω-toxins tested against contractions produced by EFS were also examined on the contraction in

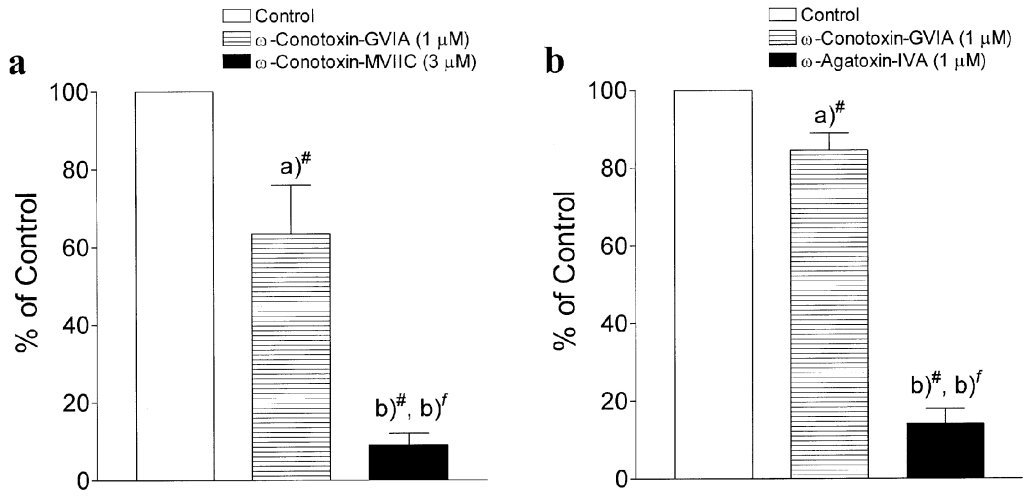

Figure 4 Inhibitory effects of ω-conotoxin MVIIC and ω-agatoxin-IVA on the ω-conotoxin-GVIA-insensitive contractile component to 32 Hz EFS. (a) Inhibitory effects of sequentially applied ω-toxins (ω-conotoxin-GVIA and ω-conotoxin-MVIIC) on the contractions of rat mesenteric artery elicited by 32 Hz EFS. ω-Conotoxin-GVIA (1 μM) and ω-conotoxin MVIIC (3 μM) were applied sequentially to the bath solution. Data are mean values ± s.e.mean of four experiments. Significant difference from the corresponding values: a)$^{\#}P<0.05$, b)$^{\#}P<0.01$ vs control response in the absence of ω-toxins; b)$^{f}P<0.01$ vs the response in the presence of ω-conotoxin-GVIA (1 μM). (b) Inhibitory effects sequentially applied ω-toxins (ω-conotoxin-GVIA and ω-agatoxin-IVA) on the contractions of rat mesenteric artery elicited by 32 Hz EFS. ω-Conotoxin-GVIA (1 μM) and ω-agatoxin IVA (1 μM) were applied sequentially to the bath solution. Data are mean values ± s.e.mean of five experiments. Significant difference from the corresponding values: a)$^{\#}P<0.05$, b)$^{\#}P<0.01$ vs control response in the absence of ω-toxins; b)$^{f}P<0.01$ vs the response in the presence of ω-conotoxin-GVIA (1 μM).

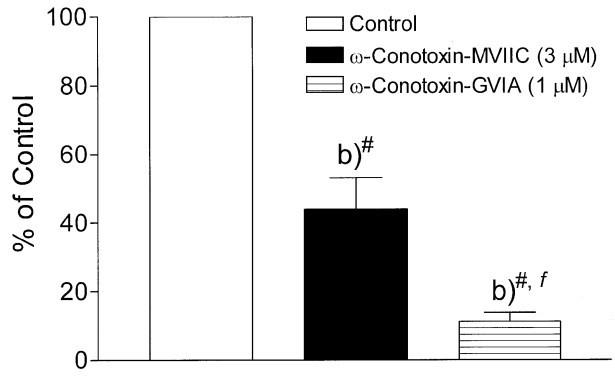

Figure 5 Inhibitory effects of single administration of ω-conotoxin-MVIIC on the contraction in response to 32 Hz EFS. ω-Conotoxin-MVIIC (3 μM) alone inhibited significantly but not completely 32 Hz EFS-evoked contraction. ω-Conotoxin-MVIIC-insensitive contractile component was strongly diminished by sequentially applied ω-conotoxin-GVIA (1 μM). Data are mean values ± s.e.mean of four experiments. Significant difference from the corresponding values: b)$^{\#}P<0.01$ vs control response in the absence of ω-toxins; b)$^{f}P<0.01$ vs the response in the presence of ω-conotoxin-MVIIC (3 μM).

response to exogenous NA. The NA-induced, concentration-dependent contraction (10 nM – 30 μM) was abolished by prazosin (100 nM), indicating that the response is mediated *via* activation of α_1-adrenergic receptors on the vascular smooth muscle cells.

Concentration-response relationships for NA-induced contraction was not affected by 1 μM ω-conotoxin-GVIA: pD_2 values of NA were not significantly different in the absence and presence of 1 μM ω-conotoxin-GVIA (7.40 ± 0.13 vs 7.34 ± 0.07, $n=4$ for each, $P>0.05$). The maximum contractile responses to NA expressed as per cent changes of the high KCl (80 mM)-induced contraction were also unchanged (103.9 ± 2.9 vs $107.4 \pm 3.3\%$, $n=4$ for each, $P>0.05$).

Combination treatment with ω-conotoxin-GVIA (1 μM) plus ω-agatoxin-IVA (100 nM) did not affect concentration-response relationships for NA-induced contraction: pD_2 values of NA were not significantly different in the absence and presence of these Ca^{2+} channel blockers (7.36 ± 0.02 vs 7.33 ± 0.04, $n=4$ for each, $P>0.05$). The maximum contractile responses to NA were also similar (130.3 ± 5.8 vs $125.7 \pm 6.0\%$, $n=4$ for each, $P>0.05$). Further addition of ω-conotoxin-MVIIC (3 μM) did not affect pD_2 values (7.08 ± 0.02 vs 7.05 ± 0.02, $n=4$ for each, $P>0.05$) and the maximum contractions (139.9 ± 9.9 vs $135.8 \pm 9.7\%$, $n=4$ for each, $P>0.05$). Even when the concentration of ω-agatoxin IVA was increased to 1 μM, combined presence of ω-conotoxin-GVIA and ω-agatoxin-IVA (1 μM for both) did not affect concentration-response relationships for NA-induced contraction: pD_2 values of NA were not significantly different in the absence and presence of these Ca^{2+} channel blockers (7.68 ± 0.13 vs 7.55 ± 0.15, $n=4$ for each, $P>0.05$). The maximum contractile responses to NA were also similar values (128.7 ± 12.9 vs $133.2 \pm 16.2\%$, $n=4$ for each, $P>0.05$). These findings show that suppression of EFS-evoked contraction of rat mesenteric artery by the ω-toxins used in the present study is not due to the direct inhibition of NA-induced contractions on vascular smooth muscle cells.

Discussion

First, we would like to emphasize that this is the first evidence which suggests the significant participation of non-N-type Ca^{2+} channels in the sympathetic nerve transmission in vascular beds.

ω-Conotoxin-GVIA is a 27-amino acid peptide isolated from the venom of the fish-hunting cone snail *Conus geographus* (Olivera *et al.*, 1984) and blocks Ca^{2+} entry through neuronal N-type Ca^{2+} channels (McCleskey *et al.*, 1987) and neurotransmitter release from nerve terminals by a pre-junctional action (Hirning *et al.*, 1988). The selectivity of

ω-conotoxin-GVIA for N-type Ca^{2+} channels is extremely high. The toxin does not affect other neuronal Ca^{2+} channels nor post-junctional L-type Ca^{2+} channels (Pruneau & Angus, 1990; Whorlow *et al*., 1996); contractile responses to exogenous NA (Pruneau & Angus, 1990; Zygmunt & Högestätt, 1993). In the present study, we also showed that contraction elicited by exogenous NA was not affected by ω-conotoxin-GVIA (1 μM). Furthermore, ω-conotoxin-GVIA (1 μM) did not affect high (80 mM) KCl-induced contraction of this artery preparation ($n=2$). Thus, suppression of EFS-induced neurogenic contraction of mesenteric artery by ω-conotoxin-GVIA (1 μM) is attributable to the inhibited release of NA due to the blockade of N-type Ca^{2+} channels in the sympathetic nerve endings.

The degree of inhibition of the neurogenic contraction of mesenteric artery by ω-conotoxin-GVIA was dependent on the frequency of EFS: The inhibition was most marked when stimulation frequency was lower ($\sim$8 Hz), which suggests the predominant role of N-type Ca^{2+} channels in the neurotransmission. Frequencies of neuronal activity was reported to be 3–4 Hz in a tonically active mammalian sympathetic ganglion (Mirgorodsky & Skok, 1969). Therefore, N-type channels may be the main neuronal Ca^{2+} channels that are responsible for the NA release from sympathetic nerve terminals in physiological conditions. By comparison, when the stimulation frequency was higher (12–32 Hz), the contraction was less sensitive to ω-conotoxin-GVIA (1 μM). Similar frequency-dependent inhibition of sympathetic and parasympathetic nerve stimulated-contractions by ω-conotoxin-GVIA was also reported by other investigators in vascular beds (Lundy & Frew, 1994; Pruneau & Angus, 1990) and bladder (Frew & Lundy, 1995).

The ω-conotoxin-GVIA (1 μM) used in the present study was 10–100 times higher than those in the previous pharmacomechanical studies (Clasbrummel *et al*., 1989; Ren *et al*., 1994; Wright & Angus, 1996; Zygmunt & Högestätt, 1993) and the maximally effective concentration for inhibition of excitatory synaptic transmission in hippocampal neurons (Wheeler *et al*., 1994). Thus, it is likely that sympathetic neuronal N-type Ca^{2+} channels in mesenteric artery tissue are completely blocked by this concentration of ω-conotoxin-GVIA. It therefore seems unlikely that frequency-related inhibition by ω-conotoxin-GVIA is due to the incomplete blockade of pre-junctional N-type Ca^{2+} channels.

In the present study, inhibition of EFS-induced mesenteric artery contraction by ω-conotoxin-GVIA (1 μM) showed wide variation. For instance, the inhibition by the ω-toxin of 32 Hz EFS-induced contraction was in the range from 5–70%. At present, we do not have any clear explanations for the reason why the inhibitory potency of ω-conotoxin-GVIA is so variable between tissues. Since we used rats weighing 200–300 g in the present study, one possible explanation would be that contribution of N-type channels to the release of NA from the sympathetic nerve terminals in mesenteric artery beds changes with development (aging) of rats and provides different mesenteric artery sensitivity to ω-conotoxin-GVIA. However, this possibility may be ruled out as a major mechanism because our unpublished observation showed that ω-conotoxin-GVIA (1 μM) inhibited 32 Hz EFS-induced contractions in a similar degree in both 5 week and 15 week rats. Other possibility would be that mechanical removal of endothelial cell layer provides different vascular smooth muscle mechanical activities in response to EFS which consequently generates wide variation in the susceptibility to the inhibition by ω-conotoxin-GVIA. In any events, clarification of variation of the sensitivity to ω-conotoxin-GVIA might lead to elucidation of the physiological significances of non-N-type Ca^{2+} channels at the sympathetic nerve terminals in vascular tissues.

To determine pharmacologically the sub-type of voltage-gated neuronal Ca^{2+} channels responsible for ω-conotoxin-GVIA-resistant contraction, putative blockers of non-N-type Ca^{2+} channels were examined on the contraction elicited by high frequency (32 Hz) EFS. The blockers tested were ω-agatoxin-IVA and ω-conotoxin-MVIIC. ω-Agatoxin-IVA is a peptide fraction from funnel web spider venom and selectively blocks P-type Ca^{2+} channels in central and peripheral neurons (Mintz *et al*., 1992a,b). ω-Conotoxin-MVIIC is a 26 amino acid peptide deduced and synthesized from a c-DNA clone derived from the marine snail *Conus magus* (Hillyard *et al*., 1992) and blocks non-selectively high threshold voltage-gated Ca^{2+} channels (N-type; P-type; and Q-type Ca^{2+} channels) (Wheeler *et al*., 1994). Neither ω-agatoxin-IVA nor ω-conotoxin-MVIIC blocks L-type Ca^{2+} channels (Olivera *et al*., 1994) or exogenous NA-induced contraction (in the present study). Thus participation of non-pre-junctional Ca^{2+} channels can be discounted in the actions of these ω-toxins.

ω-Agatoxin IVA (10–100 nM) and ω-conotoxin-MVIIC (3 μM) inhibited significantly 32 Hz EFS-induced neurogenic contraction both when the toxins were applied alone or sequentially following ω-conotoxin-GVIA. Since ω-agatoxin-IVA blocks P-type channels with an IC_{50} value of 2–20 nM (Mintz *et al*., 1992b) but also blocks Q-type channels at concentrations around 100 fold higher (Sather *et al*., 1993; Wheeler *et al*., 1994), 100 nM ω-agatoxin-IVA might exert its inhibitory actions *via* blockade of pre-junctional Q-type Ca^{2+} channels. However, a low concentration (10 nM) of ω-agatoxin-IVA also significantly inhibited neurogenic contraction in response to 32 Hz EFS, which indicates the substantial contribution of P-type Ca^{2+} channels in the response. The degree of participation of P-type channels in the neurogenic contraction based on the inhibitory action of ω-agatoxin-IVA (10–100 nM) was estimated as 10–20%. Therefore, even if the inhibitory effects by 100 nM ω-agatoxin-IVA can be totally accounted for by the blockade of pre-junctional P-type Ca^{2+} channels, activation of P-type channels present in sympathetic nerve endings seems to play a minor role in the sympathetic neurotransmission in the rat mesenteric artery.

In contrast, judging from the degree of the inhibition by ω-conotoxin-MVIIC, Q-type Ca^{2+} channels are likely to play more important roles in the release of NA from sympathetic nerve endings than N- and P-type Ca^{2+} channels especially in high frequency-stimulated contractions of mesenteric artery. Estimated contribution of Q-type channels were more than 40% to the total contraction elicited by 32 Hz EFS. Substantial role of Q-type channels has been also reported in the parasympathetic neurotransmission in the rat urinary bladder (Frew & Lundy, 1995).

In the present study, we showed that pre-junctional P- and Q- as well as N-type Ca^{2+} channels contribute substantially to the NA-mediated neurogenic contraction of rat mesenteric artery under conditions of high frequency EFS. Although physiological significance of non-N-type Ca^{2+} channels in the regulation of arterial resistance awaits further elucidation, possible co-existence of non-N-type channels together with N-type channels on individual adrenergic nerve endings would promote a high degree of flexibility in the regulation of NA release under different physiological conditions (Frew & Lundy, 1995). In spontaneously hypertensive rats (SHR), NA overflow from the sympathetic nerve terminals was reported to

be enhanced (Masuyama *et al*., 1984). Contribution of non-N-type Ca^{2+} channels to sympathetic nerve-mediated contraction in such a pathological condition might be an interesting question that should be elucidated in future.

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