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Effects of ω-conotoxin GVIA and diltiazem on double peaked vasoconstrictor responses to periarterial electric nerve stimulation in isolated canine splenic artery

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1 The actions of ω-conotoxin (ω-CTX) and diltiazem on adrenergic and purinergic components of double peaked vasoconstrictor responses to periarterial nerve stimulation have been investigated in the isolated, perfused canine splenic arterial preparation.

2 Double peaked vasoconstrictions (biphases of vasoconstrictors) were consistently observed in the conditions of 30 s trains of pulses at 1–10 Hz frequencies. ω-CTX (1–30 nM) produced similar inhibitory effects on the first phase and second phase responses in a dose-related manner. Thirty nM ω-CTX almost completely inhibited the biphasic vasoconstrictions at any used frequencies but did not affect the vasoconstrictor responses to exogenous applied ATP (0.01–1 μmol) and noradrenaline (0.03–3 nmol).

3 Intraluminal application of a large dose of diltiazem (3–10 μM) also produced a dose-dependent inhibitory effect on biphasic vasoconstrictions at any used frequencies. Three μM diltiazem exerted rather a larger inhibitory effect on the second phase than the first phase response at low frequencies (1–3 Hz), but a similar inhibition on first and second phasic responses at high frequencies (6–10 Hz). An extremely high dose of diltiazem (10 μM) almost completely inhibited the biphasic vasoconstrictor responses to nerve stimulation, and slightly inhibited the contractile responses to exogenous applied ATP (0.01–1 μmol) and noradrenaline (0.03–3 nmol).

4 The present results indicate that ω-CTX selectively acts prejunctionally to inhibit the release of transmitters from sympathetic nerve terminals, and ω-CTX-sensitive calcium channels may produce a parallel controlling of purinergic and adrenergic components of sympathetic cotransmission. A large dose of diltiazem has inhibitory effects on both prejunctional and postjunctional sympathetic co-transmission.

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Abbreviations: ATP, adenosine 5′-triphosphate; ω-CTX, ω-conotoxin GVIA; ES, electrical nerve stimulation; VGCCs, voltage-gated calcium channels

Introduction

The depolarization-induced release of neurotransmitters is dependent upon a prejunctional influx of extra-cellular calcium ions through voltage-gated calcium channels (VGCCs) (Mulkey & Zucker, 1991). N-type VGCCs have been shown to play a key role in triggering neurotransmitter release from sympathetic nerve terminals (Maggi *et al*., 1988; De Luca *et al*., 1990; Pruneau & Angus, 1990; Fabi *et al*., 1993; Ren *et al*., 1994; Wright & Angus, 1996). Since adenosine 5′-triphosphate (ATP) has been proposed as a co-transmitter with noradrenaline in peripheral sympathetic nervous system (Burnstock, 1972; 1979; 1988), an interesting point raised is whether the release of the two co-transmitters are subject to parallel modulation by N-type VGCCs. The evidences obtained in the sympathetic nerve innervating vas deferens and blood vessel preparations have shown that ω-conotoxin GVIA (ω-CTX), a selective N-type VGCC antagonist exerts a similar inhibitory effect on postjunctional responses mediated by the release of the sympathetic co-transmitter of noradrenaline and ATP (Brock *et al*., 1989; Waterman, 1997; Brock & Cunnane, 1999). However, Westfall *et al*. (1996) reported that ω-CTX reduced the nerve stimulation-evoked the release of noradrenaline to a greater extent than that of ATP in the guinea-pig vas deferens, suggesting that there is a functional separation of co-transmitter release from sympathetic nerve terminals.

L-type VGCCs appears to be involved in neurotransmitter release in sympathetic neurones (Marrion *et al*., 1987; Lipscombe *et al*., 1988). However, in the peripheral sympathetically innervated tissues, the dihydropyridine calcium channel antagonists failed to reduce the release of co-transmitters induced by nerve stimulation (Westfall *et al*., 1996; Brock & Cunnane, 1999). In contrast, diltiazem has been shown to inhibit the release of noradrenaline and the contractile responses to nerve stimulation in several blood vessel preparations (Ito *et al*., 1978; Suzuki *et al*., 1982; Zelis *et al*., 1985; Takata & Kato, 1988; Tsuda *et al*., 1990). Moreover, it is demonstrated that diltiazem at a high concentration inhibits the transmitter release because of the failure of the action potential to reach the nerve terminal, suggesting that diltiazem exerts its prejunctional action probably *via* an additional local anaesthetic action (Beattie *et al*., 1986). Recently, Yang & Chiba (1998; 1999a) reported that in the isolated canine splenic artery the double peaked vasoconstrictor responses to periarterial electrical nerve stimulation are mediated by the release of sympathetic co-transmitters noradrenaline and ATP. The present study was undertaken to compare the actions of ω-CTX and diltiazem on double peaked vasocontrictor responses to periarterial nerve stimula-

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tion in the isolated canine splenic artery, and thereby to determine the roles of N-type and L-type VGCCs in purinergic and adrenergic transmission in peripheral sympathetic nerve terminals.

Methods

Arterial preparations

Mongrel dogs of either sex, weighing 9–13 kg, were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} i.v.). After treatment with sodium heparin (200 units kg^{-1} i.v.), the dogs were killed by rapid exsanguination from the right femoral artery. The arterial main branches of the splenic artery were isolated, and side branches of the artery were tied with silk threads. Then, the artery (0.8–1.2 mm in outer diameter) was cut into segments (15–20 mm in length), and each segment was cannulated and set up for perfusion as described previously (Hongo & Chiba, 1983; Chiba & Tsukada, 1985). Briefly, a stainless steel cannula was inserted into the arterial segment from the distal to the proximal end. A proximal portion of the segment was fixed to the distal portion of a needle-type cannula with silk threads. The cannula was 3–4 cm long and 0.6–1.0 mm in outer diameter, and had small side holes 5 mm from the distal sealed end. The cannulated arterial segment was placed in a cup-shaped glass bath and was perfused by a roller pump (Tokyo Rikakikai) with Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2. The solution contained (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10. The flow rate was kept at approximately 2 ml min^{-1}. The perfusion pressure was continuously measured with an electric manometer (Nihon Kohden, MPU-0.5A) and recorded with a rectigraph (Nihon Kohden, WT-685G). After a stabilization period of 60 min, the preparation was removed from the bath solution and fixed in a horizontal position. The preparation was perfused at a constant flow rate during the experiment. The basal perfusion pressure was within 35–60 mmHg.

For electrical stimulation of the periarterial sympathetic nerve terminals, two platinum electrodes were placed on the extraluminal side of the arterial wall. Electrical stimulation was delivered by an electric stimulator (SEN-7203, Nihon Kohden) using 30 s trains of pulses at 10 V amplitude, 1 ms pulse duration, in a frequency range of 1–10 Hz. The organ bath was sealed with the plastic film to maintain the preparation at 37°C. Ten-min intervals between electrical stimulation periods were needed to obtain reproducible response. The intervals between frequency-response curves were over 60 min. The preparations were incubated for 60 min with ω-CTX and diltiazem or for 10 min with tetrodotoxin before the next response curves were made for electrical stimulation. The drug solution for ATP or noradrenaline was administered into the

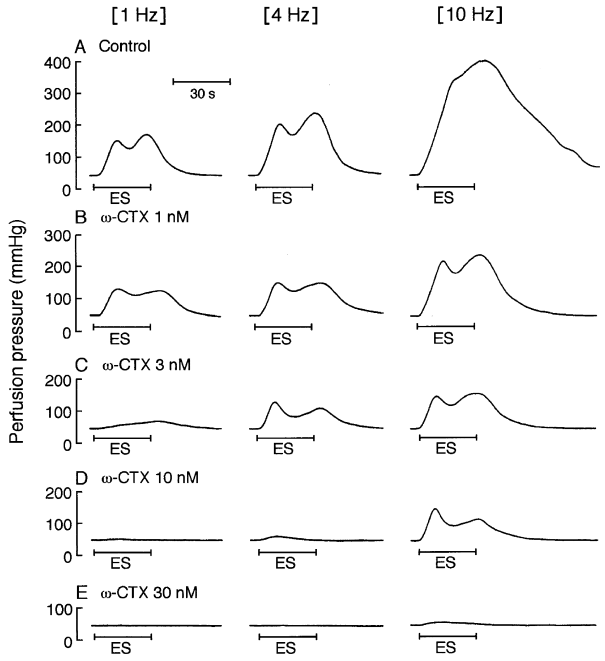

Figure 1 Double peaked vasoconstrictor responses to periarterial electrical nerve stimulation and the effects of increasing concentrations of ω-conotoxin GVIA (ω-CTX) in an isolated, perfused canine splenic artery. The vessel was electrically stimulated by 30 s trains of pulses at 10 V amplitude and 1 ms pulse duration, with a frequency of 1, 4 or 10 Hz. (ES), Electrical nerve stimulation.

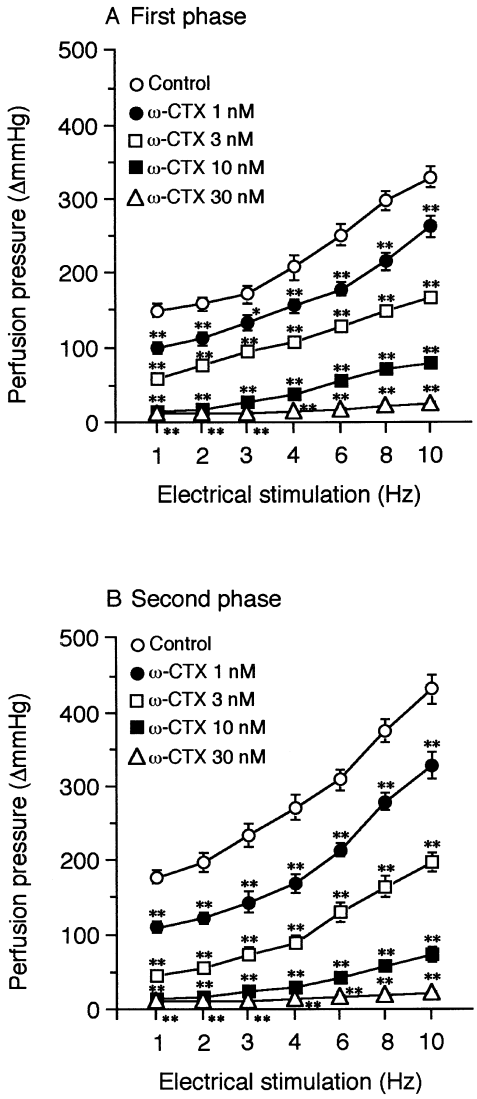

Figure 2 Effects of increasing concentrations of ω-conotoxin GVIA (ω-CTX, 1–30 nM) on the first (A) and the second peak (B) of the biphasic vasoconstrictor responses to periarterial electrical nerve stimulation (10 V amplitude, 1 ms pulse duration and 30 s trains of pulses at stated frequencies) in canine splenic arteries. Data are presented as mean±s.e.mean ($n=14$). $^{*}P<0.05$; $^{**}P<0.01$ as compared with the control group.

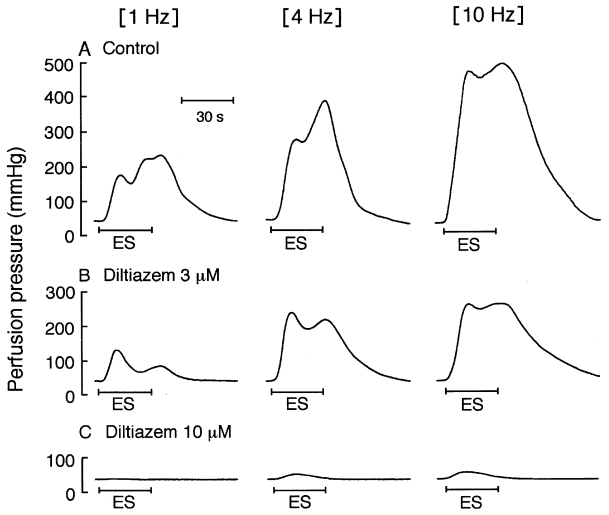

Figure 3 Double peaked vasoconstrictor responses and the effects of diltiazem in an isolated, perfused canine splenic artery. The vessel was electrically stimulated by 30 s trains of pulses at 10 V amplitude and 1 ms pulse duration, with a frequency of 1, 4 or 10 Hz. (ES), Electrical nerve stimulation.

rubber tubing close to the cannula in a volume of 0.01–0.03 ml, by use of microinjectors (Terumo, Tokyo, Japan).

Drugs

Drugs used were ω-conotoxin GVIA (ω-CTX) and tetrodotoxin (Sigma, St. Louis, U.S.A.), diltiazem hydrochloride (Tanabe Pharmaceutical Co. Ltd., Osaka, Japan), disodium adenosine 5′-triphosphate (ATP, Sigma, St. Louis, U.S.A.), *dl*-noradrenaline hydrochloride (Sankyo, Tokyo, Japan). ω-CTX was dissolved in 0.5% (w v^{-1}) bovine serum albumin in distilled water. Other drugs were dissolved in physiological saline before the start of the experiment. The stock solutions were kept at −20°C until used.

Statistical analysis

Vasoconstrictor responses to electrical stimulation are expressed as the maximal changes in perfusion pressure (mmHg) from their basal levels. The data are shown as mean ± s.e.mean. An analysis of variance with Bonferroni's test was used for the statistical analysis of multiple comparisons of data. *P*-values less than 0.05 were considered statistically significant.

Results

Vascular responses to periarterial electrical nerve stimulation

Double peaked vasoconstrictor responses (2 phases of the vasoconstriction) were readily induced with the conditions of 30 s trains of pulses at 10 V amplitude, 1 ms duration in the isolated and perfused canine splenic artery in a frequency-related manner (Figures 1A and 3A) as reported previously (Yang & Chiba, 1998). The first peak of vasoconstriction reached within 8–12 s, and the second peak within 30–35 s after the onset of electrical stimulation as shown in Figures 1A and 3A. The double peaked vasoconstrictions at used

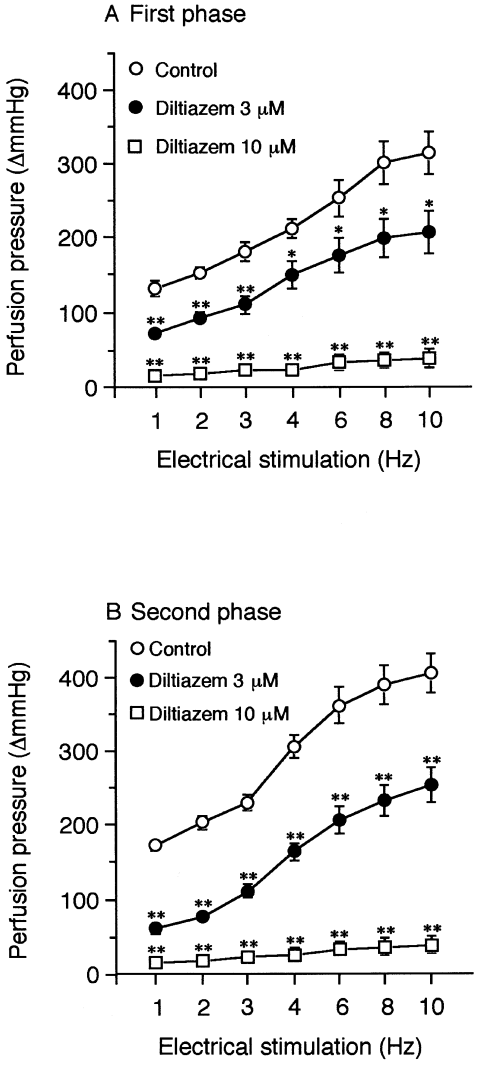

Figure 4 Effects of diltiazem (3 and 10 μM) on the first peak (A) and the second peak (B) of the biphasic vasoconstrictor responses to periarterial electrical nerve stimulation (10 V amplitude, 1 ms pulse duration and 30 s trains of pulses at stated frequencies) in the canine splenic arteries. Data are presented as mean ± s.e.mean ($n=6$). $^{*}P<0.05$; $^{**}P<0.01$ as compared with the control group.

stimulation conditions were completely abolished by the treatment with 30 nM tetrodotoxin ($n=4$, data not shown).

Effects of ω-CTX on the vasoconstrictor responses to electrical nerve stimulation

Perfusion with ω-CTX at used concentrations (1–30 nM) produced a parallel inhibition on the first phase and second phase responses to nerve stimulation in a dose-related manner (Figures 1 and 2). Figure 1 shows an original tracing of contractile force responses from typical experiments showing the effects of increasing concentrations of ω-CTX (1–30 nM). Figure 2 shows the summarized data of effects of ω-CTX on the first phase (A) and second phase responses (B). As shown in Figures 1 and 2, 10 nM ω-CTX almost completely inhibited the biphasic vasoconstrictions at low frequencies (1–3 Hz), but not at high frequencies (6–10 Hz). The biphasic vasoconstrictor responses at high frequencies were almost completely inhibited by 30 nM ω-CTX. At a high frequency of 10 Hz, after treatment with

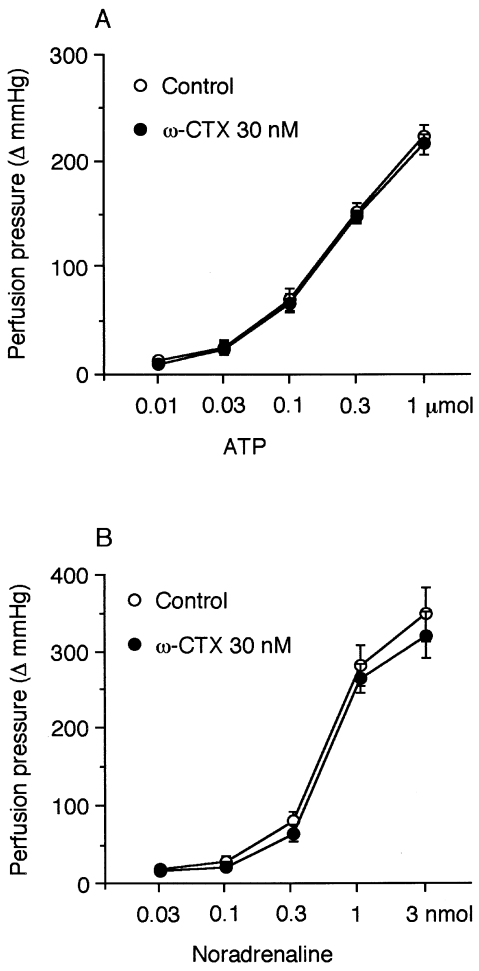

Figure 5 Effects of ω-conotoxin GVIA (30 nM) on the vascular contractile responses to ATP (A) and noradrenaline (B) in isolated, perfused canine splenic arteries. Data are presented as mean ± s.e.mean ($n=6$).

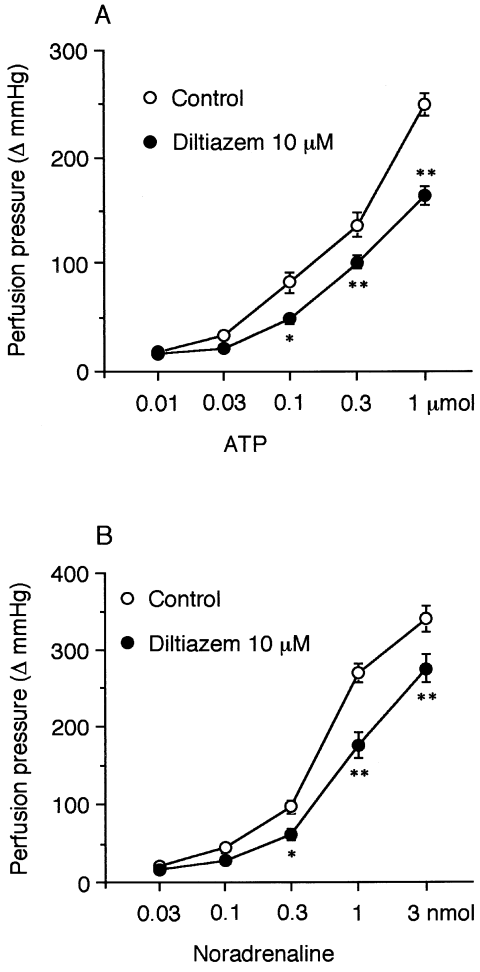

Figure 6 Effects of diltiazem (10 μM) on the vascular contractile responses to ATP (A) and noradrenaline (B) in isolated, perfused canine splenic arteries. Data are presented as mean ± s.e.mean ($n=6$). $^*P<0.05$; $^{**}P<0.01$ as compared with the control group.

30 nM ω-CTX the first and second phase vasoconstrictions were inhibited by 95% and 96%, respectively, as shown in Figure 2.

Effects of diltiazem on the vasoconstrictor responses to electrical nerve stimulation

Intraluminal application of 1 μM diltiazem did not significantly affect the vasoconstrictor responses to nerve stimulation at any used frequencies ($n=4$, data not shown). Figure 3 shows one of original tracings of contractile force, showing the effects of increasing concentrations of diltiazem (3–10 μM). Figure 4 shows the summarized data of diltiazem on the first phase responses (A) and second responses (B). As shown in Figures 3 and 4, diltiazem, at a concentration of 3 μM markedly reduced the biphasic responses at any used frequencies, and produced rather a greater inhibitory effect on the second phase than the first phase responses at low frequencies (1–3 Hz), but a similar inhibition on biphasic responses at high frequencies (6–10 Hz). As shown in Figure 4, after treatment with 3 μM diltiazem, the first and second phase responses were inhibited by 50% and 70%, respectively at 1 Hz, but by 35% and 38% at 10 Hz. Diltiazem at a high concentration (10 μM) blocked the biphasic vasoconstrictions either at low or high frequencies (Figures 3 and 4).

Effects of ω-CTX and diltiazem on the vasoconstrictor responses to administered ATP and noradrenaline

Intraluminally administered ATP (0.01–1 μmol) and noradrenaline (0.03–3 nmol) induced a dose-dependent vasoconstriction in the isolated, perfused canine splenic arteries. The vasoconstrictor responses to ATP and noradrenaline were not modified by treatment with 30 nM ω-CTX ($n=6$, Figure 5), but significantly inhibited by an administration of 10 μM diltiazem ($n=6$, Figure 6).

Discussion

The previous studies had demonstrated that ω-CTX is a potent inhibitor of N- and L-type VGCCs in sympathetic neurons (McCleskey *et al.*, 1987). However, it has been well recognized that ω-CTX inhibits the vasoconstrictor responses to electrical nerve stimulation without affecting the responses of smooth muscle cells to exogenous noradrenaline and K^+ in sympathetic nerve innervating blood vessel preparations, indicating that a proper dose of ω-CTX has no effect on postjunctional L-type VGCCs (Pruneau & Angus, 1990; Fabi *et al.*, 1993; Whorlow *et al.*, 1996).

Previously, we reported that periarterial electrical nerve stimulation readily induced a double peaked vasoconstrictions

in isolated canine splenic artery, where the first phase response might contain mainly a purinergic component, and the second response mostly an adrenergic component (Yang & Chiba, 1998; 1999a). Recently, Yang & Chiba (1999b,c) observed that the function of adrenergic component largely remained even in the cold stored preparation for 4–7 days but purinergic component of response to nerve stimulation was markedly damaged, because the first peaked responses became clearly smaller but second ones were only slightly depressed. Moreover, Yang & Chiba (1999d) also demonstrated that a smaller dose of tetrodotoxin, a selective sodium channel blocker inhibited only an adrenergic component but not purinergic component, showing that tetrodotoxin blocks second peaked vasoconstrictions induced by periarterial nerve stimulation in canine splenic artery but not first peaked ones. More recently, Yang & Chiba (1999e) demonstrated that guanethidine preferentially inhibited the second peaked adrenergic but not the first peaked purinergic responses.

Several lines of evidence obtained in the sympathetic nerve innervating tissues suggest that the release of co-transmitter noradrenaline or ATP is subjected to differential prejunctional autoinhibitions (von Kügelgen *et al*., 1994). An activation of prejunctional α_2-adrenoceptors inhibits the release of noradrenaline to a greater extent than the release of ATP (Bullock & Starke, 1990; Driessen *et al*., 1993), whereas an activation of prejunctional P1-purinoceptors exerts an opposite pattern (Driessen *et al*., 1994). Yang & Chiba (1999f) reported that treatment with an α_2-adrenoceptor antagonist, rauwolscine consistently potentiated both the first and second peaked vasoconstrictions induced by low frequencies of periarterial nerve stimulation. Moreover, an activation of prejunctional β-adrenoceptors enhances the neuronal release of noradrenaline, whereas it decreases the release of ATP (Driessen *et al*., 1996; Gonçalves *et al*., 1996). Westfall *et al*. (1996) reported that ω-CTX reduced the electric stimulation-evoked release of noradrenaline from sympathetic nerve innervating guinea-pig vas deferens to a greater extent than ATP, while a P-type calcium channel antagonist ω-agatoxin IVA did the reverse. Thus, they proposed that noradrenaline release may be more dependent on calcium influx through N-type channels whereas ATP release is coupled to calcium entry through P-type channels.

In the present study, we made an attempt to investigate whether ω-CTX exerts its action on the double peaked vasoconstrictor responses to nerve stimulation. The present results show that ω-CTX parallelly depresses the first phase and second phase responses at any used doses. Therefore, N-type VGCCs in sympathetic nerve terminals may play an important role in both purinergic and adrenergic transmission in the isolated canine splenic artery. Diltiazem has an inhibitory action on prejunctional neurotransmission mechanisms but the inhibitory potency is so small, because doses of diltiazem for inhibition are 1000 times much higher than those of ω-CTX. Moreover, as diltiazem has a postjunctionally inhibitory effect, we consider that diltiazem is not a proper substance to investigate the prejunctional mechanisms in sympathetic nerve terminals.

From these results, it is concluded that ω-CTX may produce parallel inhibitory effects on purinergic and adrenergic components of double peaked vasoconstrictor responses to nerve stimulation only *via* its prejunctional effects. An extremely high dose of diltiazem has prejunctional inhibitory action at sympathetic nerve terminals in addition to postjunctional actions.

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