Evidence for sympathetic neurotransmission through presynaptic N-type calcium channels in human saphenous vein

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1 The specific type(s) of voltage-sensitive calcium channels (VSCCs) involved in sympathetic neurotransmission have not yet been characterized in human vascular tissues. We therefore examined the functional role of the N- and L-type VSCCs in human saphenous veins.

2 Contractile response curves for transmural nerve stimulation (TNS) and for exogenously administered noradrenaline (NA) were obtained in superfused saphenous vein rings. The contractions induced by TNS, but not by NA, were inhibited by 1 μ M tetrodotoxin and by 10 μ M guanethidine. Both responses were substantially reduced by 1 μ M phentolamine, indicating that the contractions evoked by TNS were mediated by endogenous NA released from noradrenergic nerves.

3 In the presence of 2 μ M ω -conotoxin GVIA (ω -conus *Geographus* toxin, fraction VI A; ω -CgTx), a polypeptide with specific inhibitory activity on N- and L-type calcium channels, the neurally evoked contractions were almost completely abolished. In contrast, the responses induced by exogenous NA were not affected by the neurotoxin, thus providing evidence of the exclusive presynaptic action of ω -CgTx.

4 In the presence of the calcium antagonist verapamil (10 μ M), which selectively blocks L-type VSCCs, the contractions induced by both TNS and NA were diminished to the same extent, suggesting that the organic calcium blocker is active only at the postjunctional level.

5 It is concluded that N-type calcium channels are the main pathway of calcium entry controlling the functional responses induced by activating sympathetic nerves; the role of L-type channels appears to be limited to the postjunctional level, modulating smooth muscle contractions.

Keywords: Calcium channels; human saphenous veins; sympathetic neurotransmission; ω -conotoxin GVIA; verapamil

Introduction

Transmitter release from presynaptic nerve endings is regulated by the influx of extracellular calcium ions (Augustine *et al.*, 1985) flowing through voltage-sensitive calcium channels (VSCCs). Electrophysiological studies have shown that in the nervous system, VSCCs may be subdivided into L-, N-, P- and T-types, which differ in their voltage and time-dependence of inactivation as well as in their single-channel conductance (Nowycky *et al.*, 1985; Sher & Clementi, 1991). These VSCCs may also be characterized by pharmacological means (Miller, 1987). Recent patch-clamp studies have clearly demonstrated that ω -conotoxin GVIA (ω -CgTx), a 27-amino acid peptide isolated from the venom of the marine snail *Conus geographus*, is able to block N- and L-, but not T-type calcium channels at presynaptic level (Olivera *et al.*, 1984; McCleskey *et al.*, 1987; Olivera *et al.*, 1991), thus emerging as a valuable tool in the investigation of the specific type of VSCCs involved in neuronal function. Conversely, the organic Ca^{2+} channel antagonists, such as dihydropyridine compounds and verapamil, do not influence N- and T-type channels but selectively block L-channels (Miller, 1987; Ferrante & Triggle, 1990).

In sympathetic neurones of experimental animals both N- and L-channels are present (Marrion *et al.*, 1987; Lipscombe *et al.*, 1988) and appear to be involved in neurotransmitter release. N-type VSCCs have been shown to be the dominant type of Ca^{2+} entry pathway in the depolarization-evoked release of noradrenaline (NA) from rat (Hirning *et al.*, 1988) and frog (Lipscombe *et al.*, 1989) sympathetic neurones and from the sympathetic nerves in rat isolated iris (Rittenhouse & Zigmond, 1991). Furthermore, N-type channels have been demonstrated to be involved in the vasoconstrictor responses induced by sympathetic nerve stimulation in rat isolated kidney (El-Din & Malik, 1988) and in rat small mesenteric arteries (Pruneau & Angus, 1990). However, since organic

calcium channel blockers have been shown to decrease both the release of tritium and the contractile response induced by transmural nerve stimulation (TNS) in the canine saphenous vein (Jayakody *et al.*, 1986; Takata & Kato, 1988), an involvement of L-channels in sympathetic neurotransmission may also be hypothesized. Consequently, at least as far as the sympathetic innervation of the vasculature is concerned, discrepancies exist on the type of VSCCs involved in neurotransmitter release, thus suggesting that Ca^{2+} channels at noradrenergic nerve terminals probably differ in various tissues and species. In addition, although sympathetic neurotransmission in human vascular tissues has been extensively studied, the VSCCs involved in transmitter release have not yet been characterized.

The present work was undertaken to elucidate the type and the functional role of the VSCCs involved in the sympathetic neurotransmission in human vascular tissue.

A preliminary account of this work was presented at the 3rd Joint Meeting of Hungarian, Italian and Polish Pharmacological Societies, at Modena, in 1992 (Del Basso *et al.*, 1992).

Methods

Tissue preparation

Segments of macroscopically normal human saphenous veins were obtained from 41 to 60 year old male patients undergoing surgery for aorta-coronary bypass grafting. The patients were not treated with adrenoceptor agonists or antagonists or with drugs influencing the storage or release of noradrenaline. The vessel specimen was placed in an oxygenated Krebs solution at 4°C and transported immediately to the laboratory. Most of the vessels were used on the day of surgery and all the tissues were used within 18 h. The vessels

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were cleaned of the adherent connective tissue and cut into rings 4–5 mm wide. The vein segments were mounted in an organ chamber on L shaped stainless steel rods, so that it was possible to record the smooth muscle force. The preparations were superfused with Krebs oxygenated solution at 37°C by a constant perfusion pump (Gilson Minipuls II) at a flow rate of 5 ml min⁻¹; the resting tension was adjusted to 2 g and a period of 90–120 min was allowed for equilibration. The composition of the Krebs solution was (mM): NaHCO₃ 25, NaCl 118, KCl 4.7, CaCl₂·2H₂O 2.5, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.17 and glucose 5.6; the solution was aerated with a mixture of 95% O₂ and 5% CO₂. The tension of the circular muscle layer was recorded with an isometric strain gauge transducer (Grass FT 0.3 T) coupled to a polygraph (Grass 7P). TNS was delivered for 1 min through platinum wire electrodes placed on either side of the vessel. The rectangular pulses applied by an electronic stimulator (Grass S 11) were 0.3 ms in duration, of supramaximal voltage (14 V measured across the electrodes) and were applied at 1–16 Hz.

Experimental protocol

After the preparations had been allowed to equilibrate and a stable tension obtained, they were stimulated with a bolus injection of NA (40 µg), which caused near maximal contraction in pilot experiments. The contractile responses of vein rings to TNS lasting 1 min were obtained at 1, 2, 4, 8 and 16 Hz, with a period of at least 10 min between each stimulation. NA was then administered by superfusing the vessel segment for one minute with Krebs solution containing NA (0.1, 0.3, 1, 3 µM). After the control series of TNS and NA responses were elicited, antagonists were added to the super-

fusing medium and allowed to bathe the blood vessels for 30 min. A second series of TNS and NA was repeated in the presence of antagonists. Control experiments (*n* = 4; data not shown) demonstrated reproducible contractile responses to TNS and to exogenous NA in two experimental periods separated by at least 30 min. In order to normalize the data, the contractile responses were expressed as the percentage of the maximum force generated in response to 40 µg NA in each tissue.

Statistical evaluation

Data are expressed as means ± standard error of the means. Frequency-response curves to transmural nerve stimulation and concentration-response curves to noradrenaline (with and without the antagonists) were compared by analysis of variance with repeated measures (Winer, 1971). The potency-ratios were estimated according to parallel line or slope ratio assays (Finney, 1952). Results were considered statistically significant when *P* < 0.05. Confidence limits were calculated at 0.05 probability.

Drugs

(-)-Noradrenaline bitartrate, guanethidine sulphate, phenolamine hydrochloride, verapamil hydrochloride, tetrodotoxin, ω-conotoxin-GVIA were all obtained from Sigma. Noradrenaline was dissolved in 0.9% saline containing 0.1% ascorbic acid and kept in the refrigerator. Stock solutions of tetrodotoxin and ω-conotoxin were stored frozen and diluted daily in saline and discarded after use. All other drugs were dissolved in distilled water and dilutions were made in the Krebs solution.

Figure 1 Typical recordings of the effects of transmural nerve stimulation (1–16 Hz, 14 V, 0.3 ms, 1 min) and noradrenaline (NA; 0.1 µg by bolus injection) in superperfused human saphenous vein ring segments. After a control series of TNS- and NA-induced responses was obtained, drugs were added to the superfusing Krebs solution for 30 min before and during the second series of responses. Responses to TNS and NA in the absence and in the presence of 2 µM tetrodotoxin (a), 10 µM guanethidine (b) and 1 µM phentolamine (c) are shown.

Results

Characterization of the contractile responses to transmural nerve stimulation of human isolated saphenous veins

In these experiments, the results described refer to at least three vessels. As shown in Figure 1, TNS of the saphenous rings produced frequency-dependent contractile responses, which started at 0.5 Hz and reached maximum at 16–20 Hz. Since the contractions were almost completely abolished by 2 μ M tetrodotoxin and since the veins still responded to a bolus injection of 0.1 μ g NA (Figure 1a), it was assumed that the responses must be due to activation of intramural nerves. These neurogenic contractions, but not those corresponding to exogenous NA, were virtually abolished by 10 μ M guanethidine (Figure 1b), thus indicating their sympathetic nature. Adding 1 μ M phentolamine to the perfusing medium (Figure 1c) substantially reduced the contractile responses evoked by both TNS and by exogenously administered NA.

Effect of ω -conotoxin GVIA on the contractile responses to TNS and to exogenous NA

As shown in Figure 2, after the first series of TNS- and NA-induced contractile responses were performed, adding 2 μ M ω -CgTx to the perfusing medium did not cause any change in the resting tone of the vessel. However, the neurally evoked contractions decreased considerably in the presence of the neurotoxin, whereas the contractile responses to increasing concentrations of superfused NA were not affected. In 6 experiments, log frequency- and log concentration-contractile response relationships were obtained (Figure 3). In the control period, the contractile responses to TNS at all frequencies used were significantly higher than those obtained in the presence of the neurotoxin ($P < 0.001$). In the same preparations, the concentration-response curve to NA appeared to be enhanced in the presence of ω -CgTx, even though no significant differences ($P > 0.05$) were found with and without the neurotoxin.

Figure 3 Frequency- (a) and concentration-response curves (b) of the contractile responses to transmural nerve stimulation (Hz) and to noradrenaline (μ M) in the absence (●) and in the presence (○) of 2 μ M ω -conotoxin in human saphenous veins. Each point indicates the mean $\pm$ s.e.mean of 6 experiments. Contractions are expressed as a percentage of the maximum response to 40 μ g noradrenaline at the start of experiments.

Figure 2 A representative tracing from one saphenous vein ring showing the effect of 2 μ M ω -conotoxin-GVIA on the frequency-dependent contractile responses induced by 1 min transmural nerve stimulation (1–16 Hz) and on the concentration-dependent responses produced by noradrenaline (NA) superfusion. (a) Control series of responses; (b) responses in the presence of ω -conotoxin. ω -Conotoxin was added to the superfusing physiological solution 30 min before and during the second series of responses.

Figure 4 (a) A typical recording of the contractile responses evoked by transmural nerve stimulation (1–16 Hz) and by noradrenaline superfusion (NA, 0.1–3 μ M) in one superfused human saphenous vein. (b) Electrical field stimulation and NA superfusion repeated in the same vein ring after exposure to 10 μ M verapamil.

Figure 5 The effect of 10 μ M verapamil on the frequency- (a) and concentration-curves (b) of the contractile responses induced by transmural nerve stimulation (Hz) and noradrenaline (μ M). The mean responses $\pm$ s.e.mean of 6 experiments before ($\bullet$) and after ($\circ$) verapamil are shown.

Effect of verapamil

In the presence of the organic calcium entry blocker, verapamil (10 μ M), the contractile responses of the venous rings induced by both TNS and exogenous NA were reduced (Figure 4 and Figure 5). Accordingly, the neurally mediated contractions at all tested frequencies were significantly reduced by the presence of verapamil ($P < 0.01$). As in the case of TNS-induced contractions, a significant difference between the control and the second series of contractile responses carried out in the presence of the antagonist was also found for the concentration-response curves to exogenous NA ($P < 0.05$).

In order to compare the blocking effect of verapamil on the contractile responses to endogenous and exogenous NA, their relative potency-ratio was determined. Actually, the ratio of 0.16 (confidence limits 0.04–0.35) between equiactive nerve stimulations, with and without the calcium blocker, overlapped the corresponding ratio between the equiactive concentration of exogenous NA (0.31; confidence limits 0.16–0.58). This indicated that verapamil reduced equally both TNS- and NA-induced contractions.

Discussion

Even though it is now well established that different types of VSCCs are present in sympathetic neurones from experimental animals (Hirning *et al.*, 1988; Tsien *et al.*, 1988), no previous data were available on the type of VSCCs involved in the noradrenergic transmission of human vessels.

Our results show that ω -CgTx, the specific blocker of N- and L-type VSCCs, inhibits sympathetic neurotransmission in human saphenous veins without affecting the responses of the smooth muscle cells to exogenous NA. The inhibitory action of ω -CgTx on the sympathetic neurogenic responses of experimental animals has been demonstrated in the isolated kidney (El-Din & Malik, 1988), in various non vascular peripheral tissues (Maggi *et al.*, 1988) and in the vas deferens (Brock *et al.*, 1989). An inhibitory action on the responses

evoked by sympathetic stimulation, but not to exogenous noradrenaline, has already been shown in rat small mesenteric arteries (Pruneau & Angus, 1990). Hence these data, together with our results, provide direct evidence of the exclusive presynaptic action in vascular tissue of the neurotoxin, which, acting on VSCCs, probably inhibits NA release. Furthermore, these studies make it possible to define the specific type of VSCCs involved in this process. Indeed, recent studies in chick sensory neurones (Aosaki & Kasai, 1989) and in rat phaeochromocytoma PC12 cells (Usowicz *et al.*, 1990) imply a rather selective inhibition of N-type Ca^{2+} channels by ω -CgTx, although previous patch-clamp studies on chicken embryo DRG neurones demonstrated the ability of the toxin to interact with dihydropyridine-resistant, N-type channels, as well as dihydropyridine-sensitive, L-type channels (McCleskey *et al.*, 1987).

In the canine saphenous vein, the organic calcium channel antagonists verapamil, nicardipine and diltiazem have been shown to block both the contractile responses induced by TNS and NA release (Takata & Kato, 1988). Furthermore, verapamil (Godfraind *et al.*, 1986), was shown to inhibit more effectively depolarization-evoked contractions than [^3H]-

NA overflow in canine saphenous veins (Takata & Kato, 1985). In contrast to these results, calcium antagonists were unable to block [^3H]-NA overflow and contractile responses to electrical stimulation in rabbit aorta (Karaki *et al.*, 1984) or in rat isolated kidney (El-Din & Malik, 1988) and iris (Rittenhouse & Zigmond, 1991). Different types of VSCCs may therefore regulate noradrenergic transmission in a species- and tissue-specific manner.

Our study showed that the contractile responses induced by both TNS and exogenous NA were reduced by verapamil. It is therefore difficult to determine if verapamil had any prejunctional action. We found, however, that responses to TNS and NA were depressed to a similar degree, suggesting a postjunctional action only.

In conclusion, the present work provides evidence that in human saphenous veins ω -CgTx sensitive Ca^{2+} channels, probably N-type VSCCs, are present at the presynaptic level and modulate the vasoconstrictor responses to sympathetic activation by regulating the release of noradrenaline. Verapamil-sensitive L-type Ca^{2+} channels are functionally active only at the postjunctional level.

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